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FEATURES OF ADULT NEURAL PROGENITOR CELLS

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“The question is not what you look at, but what you see.”

Henry David Thoreau

To my mother, father and family

ABSTRACT

The adult Central Nervous System (CNS) harbors neural progenitor cells (NPCs) in three areas: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone in the hippocampus and around the central canal in the spinal cord. The NPCs can be isolated and cultured *in vitro*. To improve recovery after a CNS trauma by using endogenous NPCs as well as by NPC transplantation, it is important to understand the features and localization of the NPC populations. It is crucial to understand the effects of inflammatory mediators on NPCs since neuroinflammation is involved in many CNS conditions such as trauma, neurodegenerative disorders, stroke and infections. The aim of this thesis was to study different NPC features: 1. How the NPCs transcriptionally and functionally differ throughout the neuroaxis, 2. If and how inflammation effects NPCs and 3. If human Filum Terminale harbors NPCs.

I. NPCs express TLR receptors and can following activation of the receptors produce TNFα.

Toll like receptors (TLR) are involved in the innate immune system which constitutes the first line of defense against pathogens. TLR2 and TLR4 were detected in NPC cultures and *in vivo* in the SVZ. Stimulation by macrophage supernatant and the cytokines IFNγ and TNFα resulted in a differentially regulated expression of these receptors on the NPCs. Moreover, TLR2 and TLR4 agonists induced expression of both mRNA and the TNFα protein which was released from NPC.

II. NPCs change fate after exposure to chronic inflammation

We used the experimental autoimmune encephalomyelitis (EAE) model to study NPCs after chronic inflammation. NPCs were isolated and cultured from SVZ, cervical, thoracic and caudal part of the spinal cord. Thereafter a global transcriptome analysis (Affymetricx Gene Chip®) was preformed paralleled by functional analysis where the NPC capacity to differentiate was determined using immunhistochemistry and western blot. In healthy situations significant changes was found between SVZ and spinal cord- derived NPCs. SVZ NPCs had a more neurogenic fate and NPC from spinal cord was more prone to astroglial differentiation. After inflammation spinal cord NPCs transcriptional profile was altered in functions such as myelination and survival of oligodendrocytes, several canonical pathways involved in gliogenesis was downregulated. This was translated into functional fate of the spinal cord NPCs with decreased oligo-and astrogliogenesis and increased neurogenesis. SVZ NPCs after inflammation fate was skewed towards astroglia.

III. NPCs are affected by a distant on-going inflammation

In this paper we focus on NPCs from levels within the EAE-affected spinal cord which did not show signs of high level of inflammation. NPCs from spinal cord revealed an altered transcription and differentiation pattern *in vitro.* which were independent of the level of active inflammation. We also detected an increased proliferative capacity of the NPCs after inflammation in the thoracic part.

IV. Human Filum Terminale harbors NPC which can be isolated and propagated

We here characterize and describe the existence of NPCs and their distribution in Filum Terminale immunohistochmically. NPCs were also isolated and differentiated *in vitro*. After addition of growth factor NPCs displayed increased neurogenesis. We also detected an age-related difference in growth and proliferation capacity which were higher in NPCs derived from young individuals.

In conclusion, we demonstrated that NPCs differ in neurogenic and gliogenic potential depending on their origin in the healthy situation. After chronic inflammation we found that NPCs fate is altered. We also present that NPC in the SVZ express TLR receptors and can produce cytokines after inflammatory stimuli. These findings may increase the knowledge how inflammation alters the NPC fate and their regenerative potential. In human Filum Terminale harbors NPCs ressembling NPC from other CNS locations. Hypothetically Filum Terminale could be a potential cell replacement source.

Key words: adult neural progenitor cell, filum terminale, neuroinflammation, gliogenesis, neurogenesis, gene expression, spinal cord, ependymal layer, subventricular zone

LIST OF PUBLICATIONS

This thesis is based on the following studies, which will be referred to in the text by their Roman numerals:

I. Ruxandra Covacu, Lisa Arvidsson, Åsa Andersson, Mohsen Khademi, Helena Erlandsson-Harris, Robert A. Harris, Mikael Svensson, Tomas Olsson, and Lou Brundin. **TLR activation induces TNF-α production from adult neural stem/progenitor cells.** *J Immunology, 2009,183(11), 6889-6895*

II. Ruxandra Covacu*****, Cynthia Pérez Estrada*****, Lisa Arvidsson*****, Mikael Svensson, and Lou Brundin. **Change of fate commitment in adult neural progenitor cells subjected to chronic inflammation.** *Submitted manuscript*

III. Lisa Arvidsson, Ruxandra Covacu, Cynthia Pérez Estrada, Sreenivasa Sankavaram, Mikael Svensson and Lou Brundin. **Altered gene expression and differentiation in spinal cord neural progenitor cells after exposure to low level inflammation.** *Manuscript*

IV. Lisa Arvidsson***,** Michael Fagerlund*****, Nasren Jaff, Amina Ossoinak, Katarina Jansson, Anders Hägerstrand, Clas B. Johansson, Lou brundin and Mikael Svensson. **Distribution and characterization of progenitor cells within the human filum terminale.** *PLoS One 2011, 6(11):e27393*

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CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen presenting Cell
bFGF	Basic Fibroblast Growth Factor
BrdU	5´-bromodeoxy-uridine
BMP	Bone Morphogenetic Protein
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DNA	Deoxyribonucleic Acid
EAE	Experimental Autoimmune Encephalomyelitis
EGF	Epidermal Growth Factor
FT	Filum Terminale
GFAP	Glial Fibrillary Acidic Protein
IL	Interleukin
LIF	Leukemia Inhibitory Factor
INF	Interferon
LPS	Lipopolysacharide
MHC	Major Histocompability Complex
MOG	myelin oligodendrocyte glycoprotein
MS	Multiple Sclerosis
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear Factor kappa B
NO·	Nitric Oxide
NPC	Neural Progenitor Cell
PDGF	Platelet Derived Growth Factor
RA	Retinoic Acid
RMS	Rostral Migratory Stream
RNA	Ribonucleic Acid
SGZ	Subgranular Zone
Shh	Sonic Hedgehog
SOX	Sry-containing HMG box

SVZ	Subventricular Zone
TCS	Tethered Cord Syndrome
TNF	Tumor Necrosis Factor
TLR	Toll Like Receptor
Wnt	Wingless-related MMTV

1 INTRODUCTION

Few groups have described the organization of spinal cord neural progenitor cells (NPCs) around the central canal and how they react upon inflammation. When I started my PhD studies in 2006, the knowledge was scarce on NPCs in the spinal cord. There is still not to date an achievement of a full consensus on the nature of the "true stem cell" in this region. Neither was it known that filum terminale (FT) harbors NPCs.
In the western world, there are two major causes of neurological disability in young adults; trauma and multiple sclerosis (MS). These two conditions share inflammatory mediators accountable for nerve-cell damage. Neither in MS nor traumatic injury there is today a treatment to restore the injured neural tissue completely nor an understanding why regeneration of healthy tissue is so poor.
This doctoral thesis will focus on features of endogenous adult NPCs mainly derived from the spinal cord but we also studied NPCs from subventricular zone (SVZ) in the brain and filum terminale (FT). We have studied the NPCs cultured *in vitro* and their responses to Toll-like Receptor (TLR) activation. We have also investigated NPCs behavior in different parts of the normal spinal cord, reaction upon inflammation and how they differ from NPCs derived from SVZ. We choose to study NPCs in an animal MS-model where inflammation mainly occurs in the spinal cord. FT derived NPCs were mainly studied in human tissue on materials obtained from surgery. This tissue would otherwise have been discarded.

1.1 STEM CELL

Stem cells have the capacity to continuously renew and generate progeny of cells which can differentiate into various cell types. These cells have been described in plants, fungi and animals. These stem cell properties seem to be a basic feature which all multicellular organisms have in common (Weigel et al. 2002, Li et al. 2005, Thorpe et al. 2008). Lately the knowledge in this field has taken the stem cells closer to the patient.
In year 2012, John B. Gurdon and Shinya Yamanaka were awarded with the Nobel Prize in medicine for their findings that mature cells can be reprogrammed to become

pluripotent stem cells, i.e. immature cells that are able to develop into all types of cells in the body (Gurdon 1962, Takahashi et al. 2006). This finding revised the knowledge that the mature cell is static and cannot return to its immature state which had been a consensus since the existence of stem cells was experimentally proven by Till and McCulloch. They studied hematopoietic stem and progenitor cells already in 1961 (Till et al. 1961). Another Nobel Prize winner (2010) Rober G. Edwards studied embryonic stem cells in the 1960s and explanted inner cell masses from blastocysts which outgrew into many cellular lineages in culture (Cole et al. 1966). The ability to culture embryonic stem cells potentiated the generation of genetically modified mice (Gardner 1968) and later led to the development of *in vitro* fertilization in human (Steptoe et al. 1978).

In the embryo and in the adult mammal there are different types of stem cells. The hallmark features of stem cells are multipotency and unlimited self-renewal capacity (Becker et al. 1963, Siminovitch et al. 1963). After two or three divisions totipotency is lost and the cells become a morula thereafter a pluripotent blastocyst. The inner cell mass of the blastocyst contains pluripotent embryonic stem cells, which can give rise to all cell types in the body. At a later stage multipotent stem cells are formed and these generate more linage-restricted tissue types. Self-renewal is essential to keep the stem cell population alive and multipotency to give rise to differentiated daughter cells. To test if the cell have unlimited self-renewal capacity is an impossible task. Somatic cells can go through up to 80 cell divisions (Hayflick 1974) and if a cell can proliferate more than 160 times it is called “extensive proliferation”. Embryonic and neural stem cells have the capacity to go through this “extensive proliferation” (Melton DA 2009). The fertilized egg, zygote, is totipotent and may be called “the true stem cell” which is not totally in line with the definition of a stem cell due to its limited self-renewal capacity.

1.2 NEURAL STEM CELL

With the knowledge of today we know that neural progenitor cells (NPCs) are situated in 3 areas of the CNS: the SVZ, dentate gyrus subgranular zone and throughout the central canal in the spinal cord (Lois et al. 1993, Weiss et al. 1996, Johansson et al. 1999). Neural stem cells can either divide through asymmetric division which produce one differentiated cell and one new stem cell or through symmetrical division which gives two identical cells. A neural stem cell is multipotent and can with the knowledge

of today only generate neurons, astrocytes and oligodendrocytes in the CNS (Reynolds et al. 1996). In this doctoral thesis I focus on adult NPCs. "Adult" is by definition when an individual becomes sexually mature. CNS development also occurs postnatally which is the period after birth but before sexual maturation. In our study of human NPCs, cells were obtained from both adults and children.

The understanding of dividing cells and what cell types they give rise to has been debated since the start of the twentieth century when the first document regarding dividing cells in the CNS was written by Hamilton in 1901 (Hamilton 1901). Hamilton demonstrated dividing cells in SVZ and spinal cord in 4 days old rat. In 1912 Allen wrote the first article about cell division in adult rodent brain in the ventricular and SVZ up to 2 years of age (Levi 1898, Allen 1912). In spite of these findings a consensus prevailed claiming that the adult mammalian brain was without self-renewal capacity (Cajal 1913). About 50 years later, when researchers started to use radioactive thymidine ([^{3}H]thymidine) to demonstrate dividing cells, the view of the cells proliferative capacity was changed. Using this method Adrian and Walker demonstrated [^{3}H]thymidine incorporation in normal and injured spinal cord in the early sixties (Adrian et al. 1962). At this time Smart presented data of proliferation in the SVZ (Smart 1961). Altman continued in this field and reported neurogenesis in the cortex (Altman 1962, Altman 1963), hippocampus (Altman et al. 1965, Altman et al. 1966), olfactory bulb and SVZ (Altman et al. 1966, Altman 1969). 15 years later, Kaplan demonstrated adult neurogenesis in the olfactory bulb and hippocampus by visualizing the [^{3}H]thymidine incorporation in cells using electron microscopy (Kaplan et al. 1977). Nottebohm confirmed adult neurogenesis with studies on canaries and found that adult-born neurons are of functional importance of song learning (Paton et al. 1984). Reynolds and Weiss isolated stem cells in adult mammalian brain and added Epidermal Growth Factor, EGF, to the cell cultures and thereby introduced the "neurosphere" into the field. A neurosphere is a heterogeneous cell aggregate which consists of NPCs and progenitors at different stages of differentiation. (Reynolds et al. 1992). Six years after that the first human studies were performed; Eriksson 1998 identified neurogenesis in adult human hippocampus (Eriksson et al. 1998) and Johansson et al. were able to isolate human SVZ and hippocampus-obtained NPCs which differentiated into all three major CNS linages (Johansson et al. 1999). Later on the rostral migratory stream in humans was presented (Curtis et al. 2007). The human SVZ has also been demonstrated to have the ability to produce neuroblasts in adulthood (Wang et al. 2011). Still though, human adult neurogenesis in the CNS is a subject of

debate (Sanai et al. 2007) and no postnatal neurogenesis was found in the human olfactory bulb using measurement of ^{14}C incorporation (Bergmann et al. 2012).
Today we know that the brain's function depends on its ability to alter its structure due to its activity (plasticity). Most of the neuroscientific research on stem cells is performed on brain-derived NPCs. The knowledge concerning spinal cord derived NPCs is rather young and started 1996 with Weiss finding of multipotent NPCs in the mammalian spinal cord. It is today well known that adult neural stem cells from the spinal cord can be isolated, expanded and differentiated *in vitro* (Shihabuddin et al. 1997). Johansson *et al*. identified the ependymal layer in the spinal cord as a pool of stem cells and described proliferation of these cells after a traumatic injury (Johansson et al. 1999). Horner *et al.* thereafter demonstrated proliferating progenitors in the ependymal layer throughout the spinal cord as well as proliferating glial progenitors in the outer circumference in the healthy spinal cord (Horner et al. 2000). In 2008 Dromard *et al.* isolated human spinal cord-derived NPCs from non-pathological organ donors. Neurospheres were formed from spinal cord tissue of these organ donors and differentiated into glial cells and neurons (Dromard et al. 2008). This was also proved by Monthe *et al.* who isolated human NPCs and transplanted these into spinal cord injury rats. The xenografts survived and had the capacity to differentiate into neurons and glia (Mothe et al. 2011).

1.2.1 Neural Progenitor Cells of the Spinal Cord

In the adult CNS, the neurogenic regions are restricted to SVZ and SGZ in the brain. A neurogenic region is defined by germinative matrix where neurons are formed not only during embryonic development and upon different kinds of stimuli, but also in adulthood as well as during normal physical conditions. Even though spinal cord derived NPCs was demonstrated to form neurospheres and differentiate into oligodendrocytes, neurons and astocytes *in vitro* there is no evidence of neurogenesis during adulthood under healthy conditions *in vivo*. Due to this the spinal cord is generally considered a non-neurogenic region.
Different groups have described the architecture of cells in the ependymal layer around the central canal in different ways but the identity of NPCs is not fully understood. It is established that cells throughout the spinal cord continue to divide in adult life (Horner et al. 2000). The ependymal cells in a spinal cord injury have been demonstrated to

proliferate and migrate to the site of the injury (Frisén et al. 1995, Johansson et al. 1999). Using genetic labeling Meletis *et al*. defined three types of ependymal cells: cuboid ependymal cells, radial ependymal cells and tanycytes (Meletis et al. 2008). These subgroups of ependymal cells has also been described using light and transmission electron microscopy in the eighties (Bruni et al. 1987). Hamilton *et al*. found in the dorsal pole of the central canal a sub-population of tanycyte-like cells which expressed markers for neural precursors and ependymal cells. They confirmed the resemblance between SVZ and spinal cord and suggested a subependymal zone containing astrocytes, oligodenrocyte progenitors and neurons (Hamilton et al. 2009). A recent paper describes the organization and cell types of the central canal epithelium in adult mice using transmission and scanning electron microscope and immunostaining. This study demonstrates that the most common cell lining the central canal has two long motile cilia (Ecc cells) which resembles E2 cells in the SVZ. Most of the cell proliferation derives from the Ecc cells and the proliferation is most common during spinal cord growth (Alfaro-Cervello et al. 2012).

A definite specific labeling method for NPCs is lacking. For example the Ecc cells expresse Vimentin, CD24, Fox1, Sox2, CD133 but is Nestin and GFAP negative. Other groups have defined NPCs as Nestin-positive (Gilyarov 2008, Sabourin et al. 2009) and/or GFAP-positive (Meletis et al. 2008, Hamilton et al. 2009, Sabourin et al. 2009). Among other NPC markers applied (Hugnot et al. 2011) is the RNA binding protein Musashi (Okano 2006) and the cell surface glycoprotein PSA-NCAM (Dromard et al. 2008). In a majority of the studies on NPCs in spinal cord the transcription factor Sox2 is used (Graham et al. 2003, Foret et al. 2010). There are several vertebrate Sox proteins expressed in many different organs during development of the vertebrate embryo (Kamachi et al. 2000). Under development Sox proteins often work in groups and are to some extent overlapping in their expression and function. The Sox protein acts in a unique way of binding to and bending DNA. Most transcription factors bind to the major groove of the DNA but Sox protein bind to the minor groove (Ferrari et al. 1992). Expression of Sox2 has been associated with inhibition of neuronal differentiation and maintenance of progenitor state (Avilion et al. 2003, Bylund et al. 2003). Mice lacking the Sox2 gene do not develop epiblast cells (Avilion et al. 2003, Ferri et al. 2004). The expression of the Sox2 protein (and Sox1 and Sox3) is of major importance in maintaining the NPCs in a stem cell state. Expression of Sox after development can be used to reprogram somatic cells into pluripotent cells. Introducing

four transcription factors where Sox2 was one of them and the others were Klf4, c-myc, Oct-3/4, Takahashi and Yamanaka converted fibroblast cells into iPS (Takahashi et al. 2006).

1.2.2 Neural Progenitor Cells of the Subventricular and Subgranular Zones

In this thesis we used NPCs mainly derived from spinal cord but also from subventricular zone (SVZ) which is the largest pool of NPCs in the rat brain (Lois et al. 1993, Morshead et al. 1994). The SVZ is facing the ventricle and consist of four main celltypes, the neuroblast (Type A cells), SVZ astrocytes (Type B cells), immature precursors (Type C cells) and ependymal cells (Doetsch et al. 1997). The Type A cells proliferate and migrate via the rostral migratory stream (RMS) to the olfactory bulb in rodents (Doetsch et al. 1996). In the olfactory bulb the neuroblasts differentiate into GABAergic interneurons, which has also been shown in humans (Bedard et al. 2004, Curtis et al. 2007). Type B cells are a slowly proliferating group of astrocytes and type C cells are rapidly dividing precursors. The type B cell was demonstrated to generate neurospheres *in vitro* which may be differentiated into neurons, oligodendrocytes and astroglia cells. *In vivo* the type B cells are able to proliferate and differentiate firstly into type C and thereafter type A cells. These stem cell properties qualifies the type B cell to be the "true" stem cell of the SVZ(Doetsch et al. 1999). The ependymal cells of the SVZ are ciliated but also type B cells have been demonstrated to have apical processes which orient through the ependymal layer in contact with the CSF (Kokovay et al. 2012). Type A, B and C cells are situated in the NPC niche but also microglia, extracellular matrix and blood vessels also contribute to the SVZ niche.
Another neurogenic region in the adult brain which was not studied in this thesis is the dentate gyrus of the hippocampus with its subgranular zone (SGZ).The dentate gyrus is involved in forming memories, learning and localization and it is very plastic. Each day SGZ derived NPCs in an adult rat are capable to generate 9000 new cells (50% of these are neurons) (Cameron et al. 2001). In the SGZ proliferation, differentiation and migration take place during normal physiological conditions throughout life and can be altered by for example hormones, physical exercise, enriched milieu, drugs, disease, age and stress (Cameron et al. 1998, Eriksson et al. 1998, Kempermann et al. 1998, Tanapat et al. 1999, van Praag et al. 1999, Kempermann et al. 2003, Koo et al. 2008, Encinas et al. 2011, Ming et al. 2011, Boldrini et al. 2012).

1.2.3 Proliferation, Differentiation and Migration of Neural Progenitor Cells

To be able to maintain the NPC population, NPCs need to self-renew. This requires mitogens both *in vitro* and *in vivo* and without them the NPCs will differentiate. A few of these mitogenes in this thesis will be presented here: EGF, Basic Fibroblast Growth Factor (bFGF), Leukemia Inhibitory Factor (LIF) and Platelet Derived Growth Factor-BB (PDGF-BB). Spinal cord NPCs themselves also express several growth factors *in vivo.* This expression of growth factors can be changed during differentiation of the NPCs and by environmental cues (Hawryluk et al. 2012).

Notch is a membrane receptor which is active during development and *Hes-1* is a gene downstream from *Notch-1*. These play a key role in NPCs proliferation and maintenance and will also be presented here.

EGF and bFGF induce proliferation, self-renewal and expansion of neural stem cells from spinal cord. EGF and bFGF binds to tyrosin kinase receptors and cyclin D2 has been suggested to be involved in NPC proliferation. Cyklin D2 promotes an early G1 cell cycle progression (Lobjois et al. 2004). The molecular mechanisms of proliferating and self renewal in NPCs is not fully understood and gene analysis following EGF/bFGF treatment have detected over 300 upregulated genes (Nieto-Estevez et al. 2013).Weiss *et al.* demonstrated that application of bFGF or a combination of bFGF and EGF is required for proliferation in different parts of the neuroaxis, and that the NPCs from the caudal part require a higher concentration of bFGF whereas proliferation of the rostral part NPCs were EGF dependent (Weiss et al. 1996). Administering EGF and bFGF increases the proliferation of ependymal cells in healthy and injured spinal cord. It also increases functional recovery after spinal cord injury (Kojima et al. 2002, Martens et al. 2002, Jimenez Hamann et al. 2005).

LIF is a member of the IL-6 cytokine family and has previously been described by Carpenter *et al.* to increase the growth rate of human embryonic brain-derived NPC and potentiate the proliferative effect of FGF in human NPCs. This effect was not found on the rodent NPCs *in vitro* (Carpenter et al. 1999). Using microarray techniques it was demonstrated that LIF withdrawal decreases gene expression of 200 genes in NPCs and it is believed that bFGF and LIF interact with each other's signaling pathways (Wright et al. 2003, Hsieh et al. 2011). Interestingly Mothe *et al.* verified that human adult spinal cord NPCs cultured on adherent substrate increased in proliferation following

LIF addition (Mothe et al. 2011). In NPCs from embryonic spinal cord tissue this effect was not found following LIF addition (Koechling et al. 2011).

PDGF has been demonstrated to be important not only for NPCs proliferation but also for neurogenesis and oligodendrogenesis. The members of the PDGF family are three tyrosine kinase receptors and five homo- or heterodimeric PDGF ligands (Johe et al. 1996, Fomchenko et al. 2007). It has previously been shown that SVZ-derived NPCs express PDGF receptors both during development and in adulthood (Fomchenko et al. 2007, Jackson et al. 2008). PDGF-BB which is used in this thesis, is demonstrated to be involved in activation of all three PDGF receptors (Williams et al. 1997) and plays an important role in proliferation and survival of NPCs and immature neurons (Johe et al. 1996, Williams et al. 1997, Erlandsson et al. 2006).

Notch-1 is expressed by progenitors and is involved in keeping the NPCs in a self-renewing progenitor state or induce differentiation into astrocytes by negative regulation of neuronal differentiation (Tanigaki et al. 2001). In mammals there are four Notch receptors and five ligands: Delta-like 1, 3, 4 and Jagged 1, 2. The Notch proteins are cell surface molecules which are proteolytically cleaved while interacting with their ligands. The intracellular part of the receptor translocates to the nucleus and interacts with the DNA which leads to activation of downstream target genes for example *Hes-1*. In turn *Hes-1* prevent transcription of proneural genes and this interferes with neuronal differentiation and keeps the cell in a stem cell state (Nakamura et al. 2000). *Notch-1* plays an important role in CNS development (Louvi et al. 2006). It is also involved in the immune system to form developing lymphocytes into T-cells. The hematopoietic precursors express *Notch-1* whose intracellular domain activates transcription of T lineage genes in collaboration with the transcription factor GATA-3, see Abbas for review (Abbas A k 2010).

When mitogens are withdrawn the NPC differentiates. *In vitro* NPCs can be manipulated in various ways to differentiate into different lineages. However, *in vivo* it is to date not fully understood what molecular mechanisms determine the choice of differentiation. In this thesis Neurogenin, β-III-tubulin, Mash-1 are used to study neuronal differentiation (Geisert et al. 1989, Gangemi et al. 2004). For glial differentiation the expression of galactocerebrosidase, GalC, oligodendrocyte marker O4 and Glial fibrillary acidic protein, GFAP, were employed. GalC and O4 are markers

for oligodendrocytes, where O4 also labels early oligodendroglial progenitors. GFAP was used for astrocytes (Dyer 1993, Eng et al. 2000).

One feature of NPCs, which is not in focus in this thesis, is their capability to migrate. NPC migration occurs during healthy condition during development and in adulthood when the neuroblast (Type A cell) in the SVZ migrate through the RMS where they turn into interneurons (Doetsch et al. 1996, Gangemi et al. 2004). Migration also takes place during pathological conditions. Many intrinsic and extrinsic factors have been revealed to regulate NPC migration. In the RMS there are cues for repulsion, attraction and regulation of NPCs to point them into the right direction. In damaged tissues chemokines and inflammatory cytokines are involved in recruitment of NPCs. To be able to respond to these migratory signals during healthy and pathological conditions adhesion molecules and molecules connected to the cytoskeleton in the NPCs are also engaged (Leong et al. 2011).

1.2.4 Neural Progenitor Cells Heterogeneity

Studies on brain-derived NPCs demonstrate that adult SGV and SVZ have regional differences in properties of neural precursor subtypes along dorsal-ventral/rostro-caudal axes (Merkle et al. 2007, Chojnacki et al. 2009, Snyder et al. 2009). It is found that NPCs in spinal cord are highly heterogeneous when it comes to form, function and regulation compared to SVZ (Petit et al. 2011). Regional differences has also been detected within spinal cord NPCs not only during development (Barami et al. 2001, Ostenfeld et al. 2002, Piao et al. 2006) but also in adulthood (Shihabuddin et al. 1997). For example, during spinal cord development, genes from the *Hox* family are region specifically expressed along the neuro axis (Carpenter 2002) which is maintained in adulthood (Sabourin et al. 2009). In 1997 Shihabuddin *et al*. were the first to demonstrate that differentiation of NPCs from cervical, thoracic, caudal and sacral part of the rat spinal cord differ in differentiation capacity (Shihabuddin et al. 1997). Later Kulbatski and Tator compared NPCs from SVZ and the cervical and lumbal part of the spinal cord. They showed that the NPCs within spinal cord vary in capacity of differentiation and after manipulation in different ways such as adding growth factors to NPC cultures (Kulbatski et al. 2009). Pfenninger *et al*. demonstrated that NPCs from spinal cord and SVZ in healthy mice were different in gene expression (Pfenninger et al. 2011). Even though isolation and cell culture conditions may have been different in

the studies above different part of the spinal cord within the same study demonstrated differences with respect to differentiation, gene expression and proliferation of the NPCs. Not only intrinsic heterogeneity will affect the NPCs but also the environmental cues, for example when adult spinal cord NPCs was transplanted to adult dentate gyrus the cells developed into region specific neurons (Shihabuddin et al. 2000). Interestingly, other studies performed on brain (Gotz 2003, Parmar et al. 2003, Weinandy et al. 2011) and in retina (Yang et al. 2002) also indicate that the environmental cues are highly regional specific and will better support NPCs derived from the same region. These environmental cues are often referred to as the NPCs niche (Doetsch 2003).

Taken together, spinal cord development is due to interaction between highly regional specific transcription factors and morphogens. The NPCs in the adult CNS has also been found to be region specific. In this thesis we studied NPC features and investigated if there was a regional heterogeneity between different parts within the adult spinal cord with respect to proliferation, differentiation and genexpression in healthy and inflamed spinal cord.

1.3 SPINAL CORD DEVELOPMENT

The ectoderm gives rise to for example CNS, PNS and the skin. The dorsal ectoderm closes and forms the neural tube. The rostral development arises with ventricle formation and arrangement of the cephalic compartments which later on forms the brain. The caudal development occurs with formation and lengthening of the neural tube and later the spinal cord. Simultaneously with the neural tube formation cells along the tube, rostro-caudal and dorso-ventral, start to acquire regional properties. These developmental steps are highly dependent on morphogens, extracellular signals in different gradients which control cell fate at a distance. Fibroblast Growth Factor (FGF), Wingless-related MMTV (Wnt), Bone Morphogenetic protein (BMP), Sonic Hedgehog (Shh) and Retinoic Acid (RA) are important morphogens in spinal cord development. Combinations of different BMP-, Wnt- and Shh gradients lead to regional expression patterns of transcription factors along the dorso-ventral axis, see Gilbert for review (Gilbert 2010). One group of transcription factor is comprised of the *Hox* genes which are expressed differently along the rostrocaudal axis and are also involved in patterning of the spinal cord (Carpenter 2002). FGF is a protein that signals through

tyrosine kinase receptor. It has often been used as a mitogen tool for maintaining neural stem state and proliferation *in vitro* (Weiss et al. 1996). The effect of FGF is inhibited by RA which is present more rostrally and promotes neuronal differentiation in the neuroepithelium (Diez del Corral et al. 2003). Wnt is active both in dorsal-ventral and rostral-caudal development and is as BMP expressed in the dorsal neural tube (Wine-Lee et al. 2004). BMP signaling can transcriptionally induce Wnt pathway and vice versa. This BMP-Wnt balance where Wnt signaling results in differentiation and is promoted by BMP leads to regional patterning and growth of the neural tube (Chesnutt et al. 2004). Shh is expressed in the ventral part of the neural tube and the notochord and promote proliferation and maintenance of progenitor cells during development (Cayuso et al. 2006). There are different ways of cross-talk between Shh and Wnt (Wilson et al. 2012) leading to proliferation, cell patterning and axon guidance. Overall, the development of spinal cord reveals a complex and region-restricted process where many signaling pathways are included.

Brain development clearly differs from spinal cord in many ways. After enclosure of the neural tube the neuroepithelial cells within the brain divide and turn into radial glia and thereafter differentiate into neurons and glia (Merkle et al. 2004) while in the spinal cord this neuroepithelial cell pool give rise directly to neurons. However radial glia are present in the spinal cord but in contrast to the brain the radial glia appear at the very end of the neurogenesis peak and form glial cells. There is also an intense oligodendrogenesis during childhood until puberty, see Gilbert for review (Gilbert 2010).

1.4 CELLS IN THE SPINAL CORD

The CNS specific cells include neurons, oligodendrocytes, astrocytes and microglia.

1.4.1 Neurons

Neurons are excitable cells which can generate or conduct electrical impulses to other cells. The membrane potential is created by transporting ions in and out of the cell through ion channels. When the membrane potential reaches a certain threshold the action potential occurs which is transferred along the myelinated axon. When the action potential reaches the synapse, calcium channels are opened followed by calcium influx that promotes release of neurotransmitters from vesicles into the synaptic cleft. The neurotransmitters interact with the specific receptors at the postsynaptic membrane which is the substrate for inter-neural communication by inhibition or excitation. Glutamate is an important excitatory transmitter and γ-Aminobutyric acid (GABA) is an inhibitory transmitter, see Kandel for review (Kandel Eric R 1991).
Previously in our lab adult human SVZ-derived NPCs have been differentiated into functional neurons. These neurons formed synapses which communicated by glutamate and GABA and the synaptic current could be blocked with transmitter-antagonists (Moe et al. 2005, Westerlund et al. 2005).

1.4.2 Oligodendrocytes

Oligodendrocytes produce the myelin sheets around the neurons which enables a higher speed of transmission. The myelin also creates a physical insulation barrier around the neuron and supplies the neuron with trophic factors. If the oligodendrocyte population is harmed as in MS and spinal cord injury several neurons will be affected due to that one oligodendrocyte can myelinate as many as 40 different axons. This is in contrast to the Schwann cell which has a similar role in the peripheral nervous system and only myelinates one axon (Dyer 1993). Terminally differentiated oligodendrocytes fail to extend new processes and subsequently remyelination must occur from oligodendrocyte progenitor cells (Franklin et al. 1997). It was recently described that oligodendrocyte progenitors can generate Schwann cells and astrocytes

after induction of experimental autoimmune encephalomyelitis (EAE) (Tripathi et al. 2010).

1.4.3 Astrocytes

Astrocytes produce multiple membrane-bound and soluble factors which control the heterogenic environment in CNS. Astrocytes constitute the majority of cells in brain and spinal cord. They have many important functions such as providing both mechanical as well as metabolic support for neurons. Astrocyte pododcytic processes form the blood brain barrier, and can produce trophic factors (for example BDNF, EGF, NGF). During pathological conditions they participate in scar formation and immune activities for example crosstalk with microglia to increase the inflammatory response (Ridet et al. 1997, Saijo et al. 2011). There are also findings suggesting that astrocytes may be region specific. Song *et al*. revealed that astrocytes from hippocampus increased NPCs differentiation towards neurons whereas astrocytes derived from spinal cord did not promote neurogenesis (Song et al. 2002).

1.4.4 Microglia

Microglial cells are distinguished from neurons, oligodendrocytes and astrocytes by their origin, function and gene expression pattern. Microglia are believed to be of hematopoietic (mesodermal) origin and during normal conditions they have a resting phenotype and "branched" shape morphology. Interestingly, it has recently been shown that microglia precursors may develop from the yolk sac and that they early in development were detected in the developing brain (Mizutani et al. 2012). Other neuron-microglia interactions have previously been described, for example, neurons support microglia to stay in a steady-state condition by expressing and secreting chemokines. Microglia are also able to support neurons via synthesis of neurotrophic factors (IGF1, BDNF, TGFβ, NGF). As the peripheral macrophages, microglia respond rapidly to trauma or infection and can phagocyte foreign materials. Microglia can locally expand in CNS by division and change phenotype into a more "amoeboid" shape. They migrate to the site of injury along a chemotactic gradient. The cells become activated and participate in both innate and adaptive immune response by expression MHC class II, production of pro-inflammatory cytokines,

nitric oxide ($NO^{\bullet}$), reactive oxygen species (ROS), chemokines and antimicrobial peptides (Saijo et al. 2011). Even though microglia have the capacity to be detrimental to inflamed neural tissue they may also act in a neuroprotective way (see table 1). De Haas *et al.* found region-specific expression of several cell-surface proteins of the microglia which may indicate different microglial phenotypes within the CNS (de Haas et al. 2008).

1.4.5 Subgroups of Spinal Cord Neurons and Glia

There are subgroups of neurons and glia in the spinal cord. I will here present some cells which may possess some NPC features or are situated around the central canal. Neurospheres can be formed from parenchyma derived cells but these spheres are often limited in their proliferation and differentiate mainly into astrocytes and oligodendrocytes (Horner et al. 2000, Yamamoto et al. 2001, Kulbatski et al. 2007). These progenitors in the parenchyma may also respond to different kind of lesions (Ohori et al. 2006). There are a variety of cells around the central canal, some of them may not appear in all species and preparations.

In the lumbal part of the rat spinal cord there are *supraependymal neurons* which also are PSA-NCAM-positive. These cells are situated within the central canal on the ependymocytes. The function of these neurons is not yet clear and the cells are only visible by special histological preparation (Sakakibara et al. 2007) .

Radial glia is an embryonic progenitor which expresses brain lipid binding protein (BLBP) and GFAP but remains in the adult white matter of the spinal cord. Recently, it was demonstrated that the adult and neonatal spinal cord radial glia express over 100 genes shared with spinal cord and SVZ NPCs. Petit *et al.* visualized the radial glia in a BLBP-EGFP reporter mouse and found that the radial glia population was distributed in an increased cervical to lumbal gradient. Spinal cord radial glia seem to be a heterogeneous population with different and transient gene expression. They can turn into astocytes and still keep some NPC gene expression pattern in healthy animal (Petit et al. 2011). The radial glia can also become mitotic and increase their expression of developmental genes during pathological situations like EAE in mice (Bannerman et al. 2007) or in traumatic spinal cord injury (Wu et al. 2005).

Another glia cell population is the *NG2 glia* which expresses the nerve/glia antigen-2, a chondroitin sulfate proteoglycan, and PDGFRa (alpha subunit of the platlet derived

growth factor receptor). Pericytes also express these markers but the pericytes are connected to the vascular site in CNS which is not the case for NG2 glia. The NG2 glia are situated both in white and gray matter. NG2 glia exist both during the development and in adulthood and are considered to be the progenitor cell of oligodendrocytes (Richardson et al. 2011). In adult CNS NG2 glia give rise to mature oligodendrocytes and has also been demonstrated to proliferate and to differentiate into astrocytes during pathological conditions (Wu et al. 2005). *In vitro* spinal cord NG2 glia cells can form neurospheres but do not differentiate into neurons (Richardson et al. 2011).

Pericytes are part of the neurovascular unit which consists of endothelial cells of the vascular wall, smooth muscle cells (in larger vessels), astrocytic end feet and microglia (juxtavascular). To this unit the basement membrane is added and covers the pericyte which makes it to a physical barrier and together with enzymes and transporters these structures form the blood brain barrier (Krueger et al. 2010). The blood brain barrier is a barrier between the CNS extra cellular environment and blood. Pericytic aminopeptidase N (pAPN) is used to detect pericytes. In EAE affected rat pAPN expression is reduced and increased blood brain barrier permeability is detected (Kunz et al. 1995). There is limited knowledge on pericyte function and origin. Dore-Duffy propose that pericytes may be a multipotent stem cell but this is not proved *in vivo* (Dore-Duffy 2008). Recently, the Frisén group presented that scars in spinal cord injury may be composed of scar-forming stroma cells produced by a subgroup of pericytes instead of astrocytes (Göritz et al. 2011). If the proliferation of these pericytes can be blocked, the site of injury may be less impenetrable and hostile to neuroregeneration.

The meninges which cover the surface of the spinal cord have also been proven to harbor cells with some stemness properties in rat. These cells were found to be Nestin and doublecortin positive, formed neurospheres and differentiated into neurons and oligodendrocytes. Futhermore the *meningeally-derived cells* increased in proliferation and started to migrate following spinal cord injury (Decimo et al. 2011).

In lower vertebrats *cerebral-fluid-contacting neurons* are common. They are also present in rats, can be PSA-NCAM-positive and have been found in neurospheres (Bruni et al. 1987, Sabourin et al. 2009).

1.5 HUMAN SPINAL CORD EPENDYMAL CELLS AND FILUM TERMINALE

Adult human spinal cord harbors NPCs. Dromard *et al*. presented evidence that cells around the central canal expressed Nestin, CD15, Sox2, GFAP and PSA-NCAM. These cells formed Nestin- and Sox2-positive neurospheres which differentiated into glia cells and neurons (Dromard et al. 2008). Human adult spinal cord NPCs was also studied by Mothe *et al.* who transplanted the NPCs into an injured rat CNS and demonstrated that the NPCs proliferated and differentiated into neurons and glia in spinal cord injured area (Mothe et al. 2011). The ependymal layer in human spinal cord seem to be more disorganized than the rodent ependymal layer. Another difference was that the central canal in human is often occluded and the area around the central canal contains few cells but more nerve fibers and GFAP filaments (Dromard et al. 2008).

Filum Terminale (FT) is the terminal end of the spinal cord and extends from the conus medullaris to the coccyx. FT is during development attached to the first segment of the coccyx and prevents movements of the spinal cord. FT is composed of one intradural and one extradural segment. Throughout the FT the central canal can disappear and reappear in distal portions. Under normal conditions the FT consist mostly of collagen bundles, elastic fibres, astrocytes and ciliated ependymal cells lining the central canal (Choi et al. 1992, Hansasuta et al. 1999, Standring 2005, Fontes et al. 2006, Hertzler et al. 2010). Moreover, some studies demonstrate that FT also harbors neurons (Choi et al. 1992). FT is clinically affected in the disorder tethered cord syndrome (TCS). In TCS FT is tethered to the coccyx which leads to a tension of the spinal cord which affect growth and posture (Iskandar et al. 2001, Pinto et al. 2002). The most common symptom of TCS is pain which can radiate to the lower limb and eventually cause paraparesis and/or bladder dysfunction. TCS can also cause cavovarus foot, length differences between legs and scoliosis (Pinto et al. 2002). Symptoms which occur in adulthood may be due to sudden movements that cause spinal cord traction for example bending movement, trauma such as motor vehicle accident and lithotomy position during childbirth (Pang et al. 1982). In patients who suffer from TCS FT is divided by microsurgical intervention. It is beneficial to perform surgery in both children and adult patients to prevent neurological deterioration. Early surgery is often recommended in the literature

(Pang et al. 1982, Iskandar et al. 2001). TCS may also occur due to developmental malformations, intradural lipoma or ependymoma which are the most common tumors in this region (Sonneland et al. 1985, Hertzler et al. 2010, Al-Omari et al. 2011). The discovery of NPCs in FT was first published by Varghese *et al*. who also presented evidence of grafting the cells and NPCs graft survival after 10 weeks in ischemic brain lesion. The transplanted NPCs also expressed human Nestin and differentiated into astrocytes (Varghese et al. 2009). The existence of NPCs in FT was also demonstrated in a recent study where the NPCs were capable to innervate choline acetyltransferase positive cells (marker for motor neuron) and form neuromuscular junctions *in vitro* (Jha et al. 2012).

1.6 NEUROINFLAMMATION

Today we know that the immune system consists of two defense approaches, the innate immunity and the acquired/adaptive immunity. The congenital innate immunity is the first line of defense acting in a non-specific manner, whereas the adaptive immunity acts in a slower and a more antigen specific way and generates life-long memory in the host. In this thesis the Experimental Autoimmune Encephalomyelitis (EAE) rat model is used. The EAE model involves both the innate and the adaptive immunity which will be presented here.

1.6.1 Innate Immune System

The immune system must be able to recognize and destroy pathogens. The first response derives from the innate immune system consisting of a set of cells and receptors such as epithelial cells, toll like receptors (TLR), circulating and tissue resident cells and the complement system. The first identification of a pathogen or damaged cell is made by pattern recognition receptors (PRR) such as TLRs, mannose receptor and scavenger receptor. Within the innate immunity there are circulating cells for example macrophages and dendritic cells that can, upon activation by cytokines, produce tissue destructive levels of nitric oxide NO·, see Abbas for review (Abbas A k 2010). The overall production of $NO^{\bullet}$ has been used as indication of the level of inflammation in MS, EAE, traumatic brain injury and other pathological

conditions (Clark et al. 1996) (Moncada et al. 1995, Brundin et al. 1999, Danilov et al. 2003). The dendritic cell was discovered in 1973 by Ralph Steinman (Steinman et al. 1973, Steinman et al. 1978). This finding described for the first time the communication between the innate and the adaptive immune response and was awarded with the Nobel Prize in 2011. The dendritic cell which is an antigen presenting cell (APC) captures antigens for example MOG and displays it for lymphocytes in the lymph nodes. By activating the lymphocytes the innate immunity is linked to the adaptive immunity.

1.6.2 Toll Like Receptors

Toll like receptors (TLR) are membrane signaling receptors which respond to endogenous and foreign stimuli from damaged or pathologic tissue and from pathogens. The discovery that TLR was linked with the immune system was made by Bruce Beutler and Jules Hoffman (Lemaitre et al. 1996, Poltorak et al. 1998) whom together with Ralph Steinman were awarded the Nobel Prize in 2011. There are 13 different TLRs identified in mice and 11 in humans (Gambuzza et al. 2011). TLR are expressed on all immune cells (most notably on macrophages, dendritic cells and B-cells) but also in neurons, astrocytes and oligodendrocytes (van Noort et al. 2009). They could either be expressed on the cell surface (TLR1, 2, 4, 5, 6 and 11) or on endosomal membranes (TLR3, 7, 8 9 and 10), see Abbas for review (Akira et al. 2006, Abbas A k 2010, Gambuzza et al. 2011). Various TLRs bind to different type of antigens for example TLR2 binds to glycolipids and lipoprotein (from bacteria and fungi), TLR4 binds LPS (carbohydrates from gram-negative bacteria) and TLR9 to DNA from virus and bacteria. The TLRs can also bind to endogenous signals like heat shock proteins and High Mobility Group Box Protein 1, HMGB1 (protein released by dying cells or activated microglia and macrophages). TLRs function as either hetero-dimers (TLR2 which forms a dimer with TLR1 or TLR6) or homo-dimers (TLR4). After binding to an antigen the TLRs become activated. This results in activation of NF-κB which in turn leads to production of proinflammatory cytokines such as IFNα, IL-6, TNF, IL-1, IL-8, see Abbas for review (Abbas A k 2010). TLRs especially TLR4 and 2 have been suggested to play a role in MS (Bsibsi et al. 2002, Li et al. 2007, Andersson et al. 2008) and EAE (Zekki et al. 2002, Hansen et al. 2006, Marta et al. 2008, Drexler et al. 2010). TLR 9 has also been presented to be involved in EAE and

MS (Prinz et al. 2006, Marta et al. 2008) and other TLRs are shown to be involved in traumatic injuries within CNS (Zhang et al. 2012).

1.6.3 Adaptive Immune System

The most important cells of the adaptive immune system are the T-lymphocytes and B- lymphocytes (T- and B-cells). These cells act via their antigen specific receptors which are numerous and differ from each other and by that they can be very specific in their recognition of antigens, see Abbas for review (Abbas A k 2010). To be able to do this the lymphocytes (both B- and T-cells) have passed through a selection process that gives central tolerance. T-cells that bind to tightly or loosely to Major Histocompatibility Class (MHC) I or II or if it binds to a self-antigen it will be deleted in the thymus. Though some self reactive lymphocytes may reach peripheral tissue, they are then often depleted or inactivated by other T-cells (Kappler et al. 1987). B-cells are selected in the bone marrow (Goodnow et al. 1989). Failure in this tolerance will result in circulating self-reactive lymphocytes which may contribute to the development of autoimmune disease. T-cell activation is the core event of the adaptive immune system activation. When the APC presents the antigen for the T-cells they start to proliferate, form clones specific for the recognized antigen. Some of these T-cells generate a life-long "memory" against the antigen and will quickly be activated and produce cytokines if this antigen in the future enters the host. This is how vaccination works.

The T-cells are activated by two signals coming from the APC. Firstly, the T-cells receptor interacts with MHC II which is an antigen delivering molecule situated on the APC. Secondly, the T-cell surface molecule CD28 must also be activated by co-stimulatory receptors on the surface of APC (B7-1 and B7-2). These two activation signals result both in activation of the APC which starts to produce cytokines and activation of naive T-cells. Depending on the local cytokine production by APCs the T cells begins to differentiate into subgroups. The main subgroups of $CD4^+$ T-cells are Th1, Th2 and Th17. In turn the subgroups of T-cells starts to produce cytokines where Th1 is known to act in a pro-inflammatory way and secrete cytokines such as TNFα and IFNγ, while Th2 is considered to be an anti-inflammatory regulator with IL4, IL5 and IL10 production, see Abbas for review (Abbas A k 2010). A cytokine

released by Th17 is IL17 which has been demonstrated to be crucial for EAE and MS pathology (Kebir et al. 2007, Stromnes et al. 2008).

1.6.4 Experimental Autoimmune Encephalomyelitis

To study endogenous NPCs in inflammation, where both inflammation and demyelination occurs, we used the experimental autoimmune encephalomyelitis (EAE) model (Storch et al. 1998). Myelin oligodendrocyte glycoprotein (MOG) was injected into DA rats to induce EAE. The MOG was mixed with incomplete Freud's adjuvant (IFA) which is paraffin oil that enables slow release of the MOG. The IFA was not supplemented with *mycobacterium tuberculosis* which is often used for EAE induction in mice (Amor et al. 1994, Adelmann et al. 1995). In this model lesions have an affinity for the cervical part of the spinal cord and optic nerve (Storch et al. 1998). The MOG is taken up by APCs such as dendritic cells and presented to T-cells in the lymph nodes. This activates the T-cells to migrate, cross blood brain barrier and get reactivated by CNS-residing APC. They then produce pro-inflammatory cytokines and attract myelin destroying immune cells such as macrophages and $NO^{\cdot}$ producing microglia (Gold et al. 2006).

1.6.5 Multiple Sclerosis

MS is a chronic, inflammatory, progressive, neurodegenerative disease of the CNS. The prevalence in Sweden is approximately 0.1-0.2% (Landtblom et al. 2002) and females are twice as often affected than men (Koch-Henriksen 1995). A dominant location of the inflammatory lesions in MS is the periventricular areas close to the regenerative area of SVZ within which the NPCs reside (Nait-Oumesmar et al. 2007, Tepavcevic et al. 2011). Similarly, in the spinal cord MS lesions tend to be central/dorsal in location, close to the zone where proliferation of the cell layers was observed. The clinical course of MS is heterogeneous and unpredictable. Most of the patients initially present inflammatory bouts defined by relapsing and remissions of the disease (Lublin 2005). The cause of MS is today unknown but several risk factors are suggested such as environmental (for example smoking and viruses) and together with genetic factors they contribute to susceptibility for disease. Nevertheless there is

evidence that MS is connected with autoimmunity for example autorecative T-cells and antibodies which are detected in MS patient's blood, autoantibodies are also present in lesions and some drugs with antibody depletion have been beneficial for some patients (Martino et al. 1999, Elliott et al. 2012). An MS patient is affected by focal lesions in the white matter and demyelination which leads to neurological impairment. Symptoms of MS are dependent on the anatomical localization of the lesion. Common symptoms are loss of sensation, visual problems, fatigue, muscle weakness, ataxia (problem with coordination and balance), pain, cognitive impairment as well as impaired mobility (Lublin 2005).

A frequent pattern of a lesion is perivascular inflammation, infiltrating T-and B-lymphocytes, monocytes and macrophages. This is followed by demyelination where the myelin sheets are destroyed by autoantibodies, complement, macrophage activation and release of various inflammatory factors such as $NO^{\bullet}$, ROS and TGFα (Lassmann 1999, Lucchinetti et al. 2000). Demyelination of the axons leads to reduced conduction of neuronal impulses and axonal damage. Even though demyelination can to some extend be repaired by remyelination the neural damage is irreversible. This decrease in neuron density and myelin loss leads to atrophy, loss of CNS volume (Edwards et al. 2007). There is no cure for MS but several new drugs such as natalizumab may change the development of the disease.

1.7 NEURAL PROGENITOR CELLS IN NEUROINFLAMMATION

That there is a cross-talk between cells from the immune system and from CNS is today evident, but how does the immune system interact with NPCs? It has been shown that NPCs and immune cells share some immune related pathways for example TLRs, production of cytokines, chemokines and trophic factors (Kokaia et al. 2012). Several groups have reported decreased neurogenesis as an effect of proinflammatory cells (microglia and macrohpages). For example intratecal injection of LPS reduced the hippocampal neurogenesis which was later increased with addition of anti inflammatory drugs (Ekdahl et al. 2003, Monje et al. 2003). It has also been reported that inflammatory cytokines, IL-1β, TNFα, IL-6 are detrimental to neurogenesis. For instance IFNγ is known to impair proliferation of NPCs *in vitro* and in SVZ *in vivo* (Pluchino et al. 2008). But there is also proof for immune cells being beneficial for

NPCs. Ziv and Wolf demonstrated that T-cells and microglia activity increased hippocampal neurogenesis in a non-pathological condition (Ziv et al. 2006, Wolf et al. 2009). Ziv *et al*. used a rat model of environmental enrichment and found that spatial learning and memory also was T-cell dependent (Ziv et al. 2006). The neuro-protection may be due to T-cell interaction with microglia and dependent of what cytokines are involved in this interaction. It can result in protection of neurons and induction of NPCs to generate both oligodendrocytes and neurons (Butovsky et al. 2006). Combining a myelin-derived peptide vaccination with NPC transplantation promoted functional recovery after spinal cord injury. The vaccine was suggested to give a Th1 response which induced the microglia to support neural survival and NPC cell renewal (Ziv et al. 2006). The proinflammatory Th1 cell can also induce NPCs death. Conversely NPCs may kill Th1 and Th17 cells in a contact-dependent manner but not affect anti-inflammatory Th2 cells (Pluchino et al. 2005, Knight et al. 2011). Microglia can not only affect development of neurons but also oligodendrogenesis. Butovsky *et al*. demonstrated that both neurogenesis and oligodendrogenesis of adult NPCs were blocked with endotoxin-activated microglia. Furthermore they showed that IL-4 or IFNγ-activated microglia induced production of oligodendrocytes and neurons respectively (Butovsky et al. 2006). It has also been demonstrated that inflammatory cytokines IFNγ, TNFα and IL-6 induce MCH expression in cell lines from different parts of the human brain and that the NPCs cytokine-response was region specific (Johansson et al. 2008).

To summarize, immune cells can be both detrimental and beneficial for NPCs. Table one summarizes the effect of different cytokines on adult NPC functions (see table 1). The effect of the immune system seems to be dependent on type of injury and to what extent the immune cells are active. There is also evidence that some cytokines may be beneficial for neurogenesis and oligoderogenesis at a certain concentration but if the concentration is increased the cytokines act in a detrimental way (Butovsky et al. 2006, Martino et al. 2011). Finally the NPCs and the immune cells share the capacity to produce soluble molecules like cytokines, chemokines, neurotrophic factors and express receptors involved in the immune response(Imitola et al. 2004, Martino et al. 2006).

Cytokines and NO·	Effect on NPCs	Region, source, experimental set-up	References
IFN-γ	Proliferation (-)	SVZ, mouse, *in vivo/vitro* EAE	(Pluchino et al. 2008)
	Neurogenesis (+)	SVZ, mouse, *in vitro*, AD	(Baron et al. 2008)
	Neurogenesis (+)	SVZ, mouse, *in vivo*	(Wong et al. 2004)
	Neurogenesis (+)	SVZ, rat, *in vitro*	(Zahir et al. 2009)
	Oligodendrogenesis (-)	SVZ, mouse, *in vitro* AD	(Baron et al. 2008)
	Astrogliogenesis (-)	SVZ, mouse, *in vitro*	(Wong et al. 2004)
IFN-α	Proliferation (-)	SGZ, mouse, *in vivo*	(Moriyama et al. 2011)
	Neurogenesis (-)	SGZ, mouse, *in vivo*	(Moriyama et al. 2011)
TNF-α	Proliferation (-)	SVZ, mouse, *in vivo/ vitro*, stroke	(Iosif et al. 2008)
	Proliferation (-)	SGZ, mouse, *in vivo/ vitro*. EP	(Iosif et al. 2006)
	Proliferation (+)	SVZ, rat, *in vitro*	(Widera et al. 2006)
	Proliferation (+)	SVZ, rat, *in vivo*	(Wu et al. 2000)
	Neurogenesis (-)	SVZ, rat, *in vitro*	(Monje et al. 2003)
	Neurogenesis (+)	SGZ, rat, *in vivo*, stroke	(Heldmann et al. 2005)
IL-1β	Proliferation (-)	SGZ, mouse, *in vitro/vitro*	(Koo et al. 2008)
	Proliferation (-)	SVZ, mouse, *in vitro/vivo*	(Kokovay et al. 2012)
	Proliferation (-)	SGZ, rat, *in vivo*,	(Gemma et al. 2007)
	Neurogenesis (-)	SVZ, mouse, *in vitro/vivo*	(Kokovay et al. 2012)
	Neurogenesis (-)	SGZ, rat, *in vivo*	(Gemma et al. 2007)
IL-4	Migration (+)	SVZ, mouse, *in vitro*	(Guan et al. 2008)
IL-6	Proliferation (+)	Spinal cord, mouse, *in vitro*,SCI	(Kang et al. 2008)
	Neurogenesis (-)	SGZ, mouse, *in vivo*	(Vallieres et al. 2002)
	Neurogenesis (-)	SGZ, rat, *in vitro*	(Monje et al. 2003)
	Astrogliosis (+)	Spinal cord, mouse, *in vivo/vitro*,SCI	(Okada et al. 2004)
IL-10	Migration (+)	SVZ, mouse, *in vitro*	(Guan et al. 2008)
IL-15	Proliferation (+)	SVZ, mouse, *in vivo/vitro*	(Gomez-Nicola et al. 2011)
	Neurogenesis (-)	SVZ, mouse, *in vivo/vitro*	(Gomez-Nicola et al. 2011)
LIF	Proliferation (+)	Spinal cord, mouse, *in vivo*,SCI	(Azari et al. 2005)
	Proliferation (+)	SVZ, mouse, *in vivo*	(Bauer et al. 2006)
	Neurogenesis (-)	SVZ, mouse, *in vivo*	(Bauer et al. 2006)
NO·	Proliferation (-)	SVZ, SGZ, rat, mouse, *in vivo*	(Packer et al. 2003)
	Neurogenesis (-)	SVZ, SGZ, rat, mouse, *in vivo*	(Packer et al. 2003)
	Neurogenesis (-)	SVZ, rat, *in vitro*	(Covacu et al. 2006)
	Astrogliogenesis (+)	SVZ, rat, *in vitro*	(Covacu et al. 2006)

Abbreviations; (-): decrease, (+): increase, SCI: Spinal cord injury, AD: Alzheimer's disease, EP: Epilepsy

Table 1. Cytokines and NO· effect on adult NPCs. In these studies different animal models and transgenic animals are used. Overall pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 and NO· inhibit neurogenesis but some of these inflammatory mediators present a dual regulation of the NPCs. In some cases this is due to concentration of cytokines, age of animal, CNS region or type of inflammatory environment. This demonstrates how sensitive and adjustable the NPCs are to these soluble factors.

2 AIMS OF THE STUDY

I. To investigate if SVZ-derived NPCs possess TLR receptors and if these can be activated to achieve cytokine production.

II. To evaluate the gene expression and differentiation capacity of NPCs isolated from different sites along the rostro-caudal CNS axis.

III. To determine the effect of chronic inflammation on NPC gene expression and differentiation.

IV. To determine if the NPC proliferation and differentiation was dependant of the level of active inflammation.

V. To evaluate if filum terminale harbors NPCs.

3 MATERIALS AND METHODS

3.1 RODENT METHODOLOGY

All animal experiments were approved by the local ethical committee on animal research and animal care was in accordance with institutional guidelines. All efforts were made to minimize suffering. The animals had food and water administered *ad libitum* and was housed in a climate-controlled environment (21±1°C) with a 12 hour light/dark cycle. Adult female Dark Agouti rats (Scanbur B&K) were used in *Paper I, II* and *III.* Adult male Sprague-Dawley rats (Scanbur B&K) were used in *Paper IV*.

3.1.1 Experimental Autoimmune Encephalomyelitis Induction and Animal Scoring (*Paper II, III*)

Recombinant myelin oligodendrocyte glycoprotein (rMOG; aa 1–125 from the N terminus) was expressed in *Escherichia coli* and purified as previously described by Amor et al. (1994). Female rats between 7-11 weeks of age were anesthetized with isoflurane (Forane; Abbott Laboratories, Abbot Park) and immunized subcutaneously with an injection at the dorsal tail base with 200µl inoculum containing 20µg rMOG in saline emulsified 1:1 with IFA (Sigma-Aldrich, St. Louis, MO). The rats were clinically assessed daily for signs of EAE from day 9 until day 42 post-immunization. The clinical symptoms were scored as follows: 0-no clinical signs of EAE; 1-tail weakness or tail paralysis; 2- hind-limb paraparesis; 3- hind-limb paralysis; 4-tetraplegia; and 5-death. The weight of the rat was also measured daily.

3.1.2 Animal Surgery (*Paper IV*)

To obtain rat filum terminale (FT) tissue (*Paper IV*) rats were deeply anesthetized using intraperitoneal injection of medetomidine (DomitorVet. 1mg/ml, OrionPharma, Orion Corporation, Espoo, Finland) 0.5mg/kg and ketamine (Ketalar 50mg/ml, Pfizer, Sollentuna, Sweden) 75mg/kg. Partial laminectomies were performed on vertebrae L1-L5 and the conus medullaris and the FT was identified. Peripheral nerve stimulation was used during surgery in order to identify nerve roots and FT which was then carefully dissected from the surrounding tissues.

3.1.3 Cell Culturing

3.1.3.1 *Neural Progenitor Cell Culturing (Paper I-III)*

Brains and spinal cords were harvested from healthy control animals and from EAE diseased animals with clinical score 2-3 *(Paper II-III)*. NPC cultures were isolated and propagated from the SVZ *(Paper I, II)*, different levels of the spinal cord *(Paper II and III*) and filum terminale (FT) *(Paper IV)*. In *Paper II* and *III* the spinal cord was divided into 3 parts which were defined as follows: cervical (above Th2) thoracic (Th2-Th12) and caudal (below Th12). NPCs were isolated in accordance to a modified protocol described by Johansson *et al*. (Johansson et al. 1999). For isolation of the spinal cord-derived NPCs, the spinal cord was divided rostro-caudally and the meninges were peeled off before mechanical and enzymatic dissociation using 200 U/ml DNAse (Sigma-Aldrich) and 10U/ml papain (Worthington). To remove myelin debris cells were resuspended in 0.9M sucrose in Hanks' Balanced Salt Solution (HBSS) (Invitrogen) and pelleted at 750g for 10 min followed by additional washing with L15 medium. The cells were cultured in 10cm Ø petri dishes in neurosphere medium, composed of Dulbecco's Modified Eagle's Medium/F-12 containing B27 supplement (Gibco), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Life Technologies, Invitrogen AB, Stockholm, Sweden, http://www.invitrogen.com) 20ng/ml epidermal growth factor (EGF, Sigma-Aldrich, Stockholm, Sweden, http://www.sigmaaldrich.com) and 20ng/ml basic fibroblast growth factor (bFGF, R&D systems). The NPC cultures were propagated and passaged with papain/DNAse twice and used in experiments as single cells after the second passage. For differentiation, single cell suspensions were seeded onto poly(D-lysine)-coated plates (Sigma-Aldrich), and cultured for 5-7 days in medium lacking EGF/bFGF but supplemented with 1% fetal calf serum (FCS) (Life Technologies). Cells were cultured with a density of 150 000 cells per petri dish.

3.1.3.2 *Macrophage Cell Culture, Cytokines and Preparation of Supernatants (Paper I)*

Femurs from DA rats 7- to 8-wk-old were collected, and the marrow was flushed out and dissociated through a 25-gauge needle according to Andersson *et al.* (Andersson et al. 2004). Cells from two femurs were pooled in a 175-cm^2 flask and cultured in DMEM supplemented with 20% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 2-ME (β-Mercaptoethanol) (all reagents from Life Technologies) and 20% L929 cell line supernatant. In total, the cells were cultured for 10 days, the last 2 days without the L929 supplement. Cells were harvested using trypsin-EDTA (Life technologies) for 10 min at 37°C, washed, and re-plated at 2 or 1 X 10^5 cells/ml. After 24 h, the cells were activated using 100 ng/ml LPS (Sigma-Aldrich) or 100 U/ml IFN-γ, a gift from Dr. P. H. van der Meide (Utrecht University, the Netherlands) for 16 h. The cells were washed twice with DMEM/F12 and cultured with stem cell medium in the absence of mitogens for further 96 h when the medium was collected, filtered, and stored at -20°C. The supernatants were diluted 1/1 (vol/vol) in fresh NPC medium with epidermal and basic fibroblast growth factors

before applying them onto the NPC cultures. Two different controls for the carry-over of stimulants were performed. The "medium control" was actually supernatant collected from a well without macrophages. The stimulants were added to the well and the medium was changed 16 h later as for the wells containing macrophages. After 96 h, the medium was collected, filtered, and added to the NSC cultures designated as medium controls. To further control for carry-over of LPS, the known inducer of the TLR2 expression, IFN, was used as the sole macrophage stimulant. This process was done to exclude that increased TLR2 expression was due to LPS contamination. The supernatant from IFN-stimulated macrophages was as affective in inducing the TLR2 expression as the one from IFN plus LPS-stimulated macrophages. The cytokines and the concentrations used in *Paper I* were recombinant rat IFN-γ (100 U/ml), a gift from Dr. P. H. van der Meide (Utrecht University, the Netherlands), and recombinant rat TNF-α (1 ng/ml; Sigma-Aldrich). The following TLR agonists were used: Pam3Cys (1 µg/ml; EMC Microcollections), LPS from *Escherichia coli* O111:B4 strain (100 ng/ml; Sigma-Aldrich), ultrapure LPS from *E. coli* O111:B4 strain (100 ng/ml; Sigma-Aldrich), lipoteichoic acid (LTA; Sigma-Aldrich), and HMGB1 (high mobility group box chromosomal protein 1, 1 µg/ml; partly purchased from Sigma-Aldrich and partly provided by Dr H. Erlandsson-Harris, Karolinska Institutet, Stockholm, Sweden). The used concentrations were determined with titration experiments and by using other researchers' publications as guidelines.

3.1.4 Flow Cytometery and Cell Sorting (*Paper I*)

Single cell suspensions of NPCs were fixed for 30 min at 4°C using 1% paraformaldehyde in PBS. Unspecific binding was blocked with 5% rat serum/0.1% saponin in PBS for 20 min. After washing, the primary Ab was applied for 30 min and washed, and the fluorophore conjugated secondary Ab was applied for an additional 30 min. The cells were analyzed using a FACSCalibur (BD Biosciences). The entire staining procedure was performed on ice. For negative sorting of $CD11b^+$ cells, the neural spheres were dissociated 24 h before sorting and cultured in propagation medium. The following day the cells were washed, blocked in 5% rat serum in PBS, and stained with CD11b Ab and respective isotype control (10 µl/million cells). The cells were then sorted using a MoFlow high-speed cell sorter (DakoCytomation). For flow cytometry the following antibodies were used (at 1/100 dilutions): goat anti-TLR2 (Santa Cruz Biotechnology), goat anti-TLR4 (Santa Cruz Biotechnology), goat IgG (Jackson ImmunoResearch Laboratories), donkey anti-goat Alexa Fluor 488 (Molecular Probes), mouse R-PE-conjugated anti-CD11b (Serotec) and IgG2a-negative control (Serotec), PE-conjugated hamster (IgG1) anti-rat/mouse TNF antibody, and PE conjugated hamster IgG1 (BD Biosciences). Isotype-matched antibodies were used to control the specificity of the primary antibody.

3.1.5 Rat Tissue Sections (*Paper I, III and IV*)

Rat CNS sections were obtained in *Paper I, III and IV*. All animals were anesthetized with an overdose 0.5 mg/kg medetomidine (Domitor Vet. 1 mg/ml, OrionPharma) and

75 mg/kg ketamine (Ketalar 50 mg/ml, Pfizer) injected intraperitoneally. Animals were perfused transcardially with body-temperature (37°C) saline followed by cold (4°C) 4% PFA (Apoteket) in PBS. The tissue was removed and post fixed for 1 h in cold (4°C) 4% PFA in PBS followed by rinse in PBS and cryoprotected for 1 h (for FT) and 3h (for brain and spinal cord) in 17% sucrose (w/v) in PBS. Brain and spinal cord were then kept in 15% sucrose in PBS for 3 days. FT was cryosectioned in longitudinal and transverse sections (10-14 µm thick) using a Leica CM3000 (Leica Microsystems). Sections were mounted on SuperFrost Plus microscope slides (Menzel-Gläser).

3.1.6 Methods for Transcriptional Studies

3.1.6.1 *Quantitative Real-Time PCR (Paper I, II and III)*

Total mRNA was isolated using an RNeasy mini kit (Qiagen) and cDNA subsequently prepared using the iScript kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR (qPCR) was performed using a BioRad iQ5 iCycler Detection System with a three-step PCR protocol (95°C for 10 min followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec), using SYBR Green (Bio-Rad). Expression levels corrected for amplification efficiency and normalized to house-keeping gene expression, were analyzed using iQ5 v2.0 software (BioRad). Primers were designed using Primer Express software (Applied Bio-Systems) (for primers applied see material and methods in *Paper I, II* and *III*). Relative mRNA quantities of the target genes and housekeeping genes were calculated using standard curves made for each primer pair. The standard curve was constructed of a 4-fold dilution series of pooled samples. For each individual sample the expression of the target gene was normalized using 2-3 housekeeping genes, *β-actin, Gapdh* and *Hprt*.

3.1.6.2 *Microarray Sample Preparation and Analysis (Paper II)*

For the microarray expression analysis only animals with disease reaching score 3 (paralysis) were used and sacrificed at 40 days post immunization. NPCs obtained from 3 control animal and 3 EAE affected animals were studied. Gene expression was measured in the following experimental groups: NPC cultures (undifferentiated or differentiated) isolated from the SVZ and the cervical, thoracic and caudal parts of the spinal cord and from the SVZ biopsy prior to NPC culturing. Within each of these groups three naïve and three EAE individuals were compared. The array platform used was Affymetrix GeneChip® RAT Gene ST 1.0; one microarray was used for each individual. Total RNA extraction and on-column DNase treatment was performed using the RNeasy mini kit from Qiagen. Further RNA quality control using an Agilent Bioanalyzer, array hybridization and basic data processing was performed at the Bioinformatics and Expression Analysis Core facility at Karolinska Institutet, Stockholm, Sweden. The basic data processing involved background signal correction using the GC composition-based background correction algorithm (PM-GCBG), array normalization with global median and signal summarization using the probe logarithmic intensity error estimation (plier), all steps performed in the GeneChip Expression console from Affymetrix. To calculate the statistical significance between

naïve and EAE groups two-sided unpaired student's t-test was used. The false discovery rate (FDR) was calculated using the q-value plugin for R and an FDR level of 5% was set. The functional analysis and canonical pathway analysis of the entire data set was generated with IPA (Ingenuity Systems, www.ingenuity.com). Molecules from the dataset that met the signal intensity cutoff of 50 or higher, and passed a 5% FDR level, were considered for the analysis. To determine the p-value of the association between the data set and a function, disease or canonical pathway Fisher's exact test and Benjamini & Hochberg correction for multiple testing was used. The ratio provided for the canonical pathway analysis is calculated by dividing the number of molecules from the data set that map to a particular pathway to *the total number* of molecules annotated/curated to the particular canonical pathway.
To identify differentially expressed genes between NPC groups from naïve animals one-way non-parametric Anova (Kruskal-Wallis test) with adjusted Bonferroni correction for multiple testing performed in Multiple Array Viewer (MeV) (Saeed et al. 2003) and Dunns test performed in GraphPad Prism 5. Functional clustering was performed in WEB-based Gene Set Analysis Toolkit (WEBGESTALT) (Duncan D.T. 2010) and/or DAVID Bioinformatics Resources 6.7 (Huang da et al. 2009).

3.1.7 Methods for Protein Detection

3.1.7.1 *ELISA Quantification (Paper I)*

Supernatants were collected from NPC cultures 48 and 96 h after stimulation with TLR agonists, centrifuged, and frozen at -20°C. TNF-α was measured using the Quantikine ELISA kit from R&D Systems.

3.1.7.2 *TNF-α Intracellular FACS Staining (Paper I)*

NPCs were seeded on poly-D-lysine-coated plates and stimulated for 48–96 h with 1 µg/ml Pam3Cys (EMC Microcollections). When 5–8 h of the stimulation time remained, brefeldin (BD GolgiPlug; BD
Biosciences) was added to the cultures, 1 µl/ml culture medium. The cells were harvested using pre-warmed 100 µg/ml Liberase Blendzyme 1 (Roche Diagnostics) in PBS. Subsequent fixation, blocking and staining steps were performed according to the BD Cytofix/Cytoperm Plus instruction manual (BD Biosciences). The PE-conjugated hamster (IgG1) anti-rat/mouse TNF Ab and matching isotype control were also purchased from BD Biosciences. To further control the staining specificity, a ligand-blocking control was performed by pre-incubating the TNF-specific Ab with rat TNF-α protein (Sigma-Aldrich).

3.1.7.3 *Immunohistochemistry (Paper I, II, III and IV)*

Cells were differentiated on poly-D-lysine hydrobromide (Sigma-Aldrich) coated glasses or coverslips, fixed with 4% paraformaldehyde in phosphate-buffer saline (PBS) (Bie&Berntsen A-S) blocked in PBS/0.1% saponin/10% goat serum and

incubated with the primary antibody overnight. For Nestin and Sox2 stainings the cells were not differentiated. After washing, the secondary antibody was applied for 1 hour at RT. Concentration and antibodies used were, rabbit anti-glial fibrillary acidic protein (GFAP) 1:1000 (Dako), mouse anti-galactocerebroside (Gal C) 1:100 (Millipore), mouse anti-β-III tubulin 1:100 (Millipore), mouse anti-CD11b 1:200 (Millipore), rabbit anti-Sox2 1:100 (Millipore), mouse anti-Sox2 1:100 (Millipore), mouse anti-Nestin 1:100 (Millipore), mouse anti-BrdU (dilution according to supplier, Amersham), rat anti-BrdU 1:50 (AbD Serotec), mouse anti-TLR4, 1:40 (Abcam), mouse IgG2a 1:100 (Dako-Cytomation), rabbit IgG 1:100 (R&D Systems), anti-rabbit IgG Alexa Fluor 488 1:100 (MolecularProbes), biotinylated rabbit anti-goat 1:200 (DakoCytomation), goat anti-rat Alexa 488 1:00 (Invitrogen) Cy3 donkey anti-mouse 1:1000 (Jackson ImmunoReseach), Alexa 488 donkey anti-rabbit 1:500 (Invitrogen) and goat anti-mouse IgG 594 1:100 (Invitrogen). For the Sox2 immunostaining the fixed cells were first permeabilized with a buffer containing 20 mM HEPES (pH 7.4), 300 mM saccharose, 50 mM NaCl, 3 mM MgCl2, 0.5% (vol/vol) Triton X-100 for 3 min at 4°C (30). Cells were then blocked and incubated with the primary Ab. For visualizing all cells the nuclei were counterstained with DAPI (4’,6-diamidino-2-phenylindole, Invitrogen).Glasses were mounted in Mowiol (Cabiochem).

The rat tissue cryosections were air-dried, washed in PBS for 10-30 min and then incubated for 60 min in PBS containing 1% BSA, 0.3% Triton-X and 0.1% sodium azide to prevent nonspecific binding. All of the primary antibodies used were diluted in this solution. Sections in *Paper I* were blocked for 1 h at room temperature in PBS/0.3% Triton X-100/4% BSA/4 drops of avidin block solution per ml (Vector Laboratories) and the primary antibodies (TLR2 or TLR4, 1:100; Santa Cruz Biotechnology) was diluted in PBS/0.3% Triton X-100/1% BSA and four drops of biotin block solution per ml (Vector Laboratories) was applied overnight at 4°C. Biotinylated secondary antibody, diluted (1:200) in blocking solution (without the avidin blocking solution), was applied and incubated at room temperature for 1 h. The ABC solution (Vector Laboratories) was applied after washing and incubated for 1 h at room temperature. The staining was visualized using a diaminobenzidine substrate kit for peroxidase (Vector Laboratories) for 5–6 min. The sections were counterstained with hematoxylin, washed in tap water, dehydrated in ethanol and mounted in DEPEX Mounting medium (VWR International).

CNS tissue sections from *Paper III* were first rinsed in PBS followed by incubation with 0.1M NaOH for 2 minutes and then in PBS pH 8.5 for 30 seconds. Sections were then incubated in primary anti-BrdU according to standard protocol from the supplier and Sox2 antibody was added as previously described. Sections in *Paper IV* were incubated with the primary antibody mouse anti-Nestin (dil. 1:100, Chemicon) for 24 h at +4°C, rinsed in PBS and subsequently incubated with species-specific secondary antibodies diluted in PBS; Alexa 488 donkey anti-mouse 1:500 (Molecular Probes/Invitrogen). Sections in *Paper I* and *IV* was also stained with hematoxylin/eosin. All sections were counterstained with nuclear marker DAPI (Molecular Probes, Invitrogen). Tissue was mounted in Mowiol (Calbiochem, VWR International).

3.1.7.4 *Western Blot (Paper II)*

NPCs were seeded into six-well plates at a density of 2 million cells per well in differentiation medium for 5-7 days. The cell homogenates were mixed with sample buffer and heated to 100°C for five minutes. Cell homogenates were separated by SDS-polyacrylamid gel electrophoresis (PAGE) at 180V, and transferred to a nitrocellulose membrane (1h at 100V). After blocking in PBS/Tween (0.01%) with 5% non-fat milk, the membrane was incubated with primary antibody at 4°C over night. After washing the secondary antibodies were added to the membrane for 1 h at RT. Antibodies used were rabbit anti-glial fibrillary acidic protein (GFAP) 1:1000 (Dako), mouse anti-galactocerebroside (Gal C) 1:300 (Millipore), β-actin 1:2000 (Abcam), mouse anti-tubulin βIII isoform (Tuj) 1:50 (Millipore), swine anti-rabbit HRP 1:500 (Dako) and goat anti-mouse 1:1000 (Dako). Bands were detected using an enhanced chemiluminescence Western Blotting Detection kit (GE Healthcare Bio-Sciences AB http://www1.amershambiosciences.com/). Thereafter the bands were scanned (Umax PowerLook 1120) and the net intensity was measured using the ImageJ software (http://rsbweb.nih.gov/ij/).

3.1.8 Methods for Detection of Cell Proliferation

3.1.8.1 *[^{3}H]Thymidine Incorporation (Paper I and III)*

NPCs were seeded on 96-well U-bottom plates in duplicates-triplicates 25000 (*Paper III*) or 50000 (*Paper I*) cells/well in 200 µl of culture medium with or without epidermal and basic fibroblast growth factors. In *Paper I* the cells were exposed to TLR agonists for 48 h before pulsing with [^{3}H]thymidine (1 µCi/well) for 24 h. In *Paper III* cells were pulsed with [^{3}H]thymidine for 48h without TLR agonists. Cells were thereafter harvested using a Tomtec cell harvester (PerkinElmer Wallac, Turcu, Finland, http://www.perkinelmer.com). The incorporated radioactivity was measured using a β-liquid scintillation counter, 1450 Microbeta Plus (PerkinElmer Wallac).

3.1.8.2 *BrdU Incorporation (Paper III and IV)*

BrdU (5-bromo-2'-deoxyuridine) (50mg/kg in PBS, Sigma, St Louise, MO, USA) which was used *in vivo* in *Paper III* was administered subcutaneously once daily for 10 days starting from onset of neurological signs for EAE. Animals were clinically assessed daily for signs of EAE. Animals were perfused through intracardial infusion with body warm isotone saline and thereafter ice-cold formaldehyde (4% w/v). The spinal cord was dissected and postfixed in formaldehyde (4%) and then rinsed in PBS, and immersed in sucrose (15% w/v) overnight. Transverse sections were prepared (14 µm thick) using a Leica CM3000 (Leica Microsystems) and mounted on SuperFrost® Plus microscope slides (Menzel-Gläser, Braunschweig, Germany).
BrdU was also used *in vitro* in *Paper III* and *IV*. After passage the NPC neurospheres or single cells were pulsed with BrdU (Sigma) for 48 h in the presence of EGF and bFGF. The spheres were placed on coated glasses and before fixation in 4% PFA. The glasses were stained as previously mentioned.

3.1.9 Colorimetric Measurement of Nitrite (*Paper II and III*)

Nitrite can be used as a marker for inflammation. We wanted to investigate which spinal cord parts contained inflammatory lesions and define inflamed parts of the EAE affected animals above cut-off which was calculated based on the normal distribution of the nitrite levels in controls. The cut-off for inflamed parts was set to values equal or above mean+2SD. Nitrite measurements below this level was identified as areas with low grade inflammation. Supernatants from NPC cultures after their first passage was collected into eppendorf tubes previously washed with nitrite-free water and autoclaved and stored in -20 until use. The nitrite levels in these supernatants were measured utilizing Griess reaction (Griess 1864). The supernatants were mixed with Griess Reagent (Sigma-Aldrich) 1:1 in 96-wells ELISA plates and incubated for 15 min at room temperature (RT). The absorbance was read at 562 nm using an ELISA reader (EMax Precision Microplate Reader, Molecular Devices). The standard curve was constructed from a nine-fold dilution series of sodium nitrite dissolved in stem cell medium.

3.2 HUMAN MATERIAL

The human study *(Paper IV)* was approved by the Stockholm county ethical committee for human research and carried out in accordance with the Helsinki declaration. The patient or the parents of the patient was informed in accordance with the ethical approval and tissue was obtained after informed consent by patient or parent. Only tissue from Karolinska University Hospital was used.
There were 21 donors used, both young and adult patients (1-60 years). MRI was performed on the patients to exclude tumors; the patients were also screened for infectious disease before the surgery procedure. Immediately after resection of FT in the operating room the tissue was stored in L15 medium (4°C) and instantly transported to the laboratory. Part of FT tissue from patients was fixed in ice-cold 4% PFA (24 h) and cryoprotected in 17% sucrose before being imbedded in Cryomount (HistoLab Gothenburg). FT was longitudinally and/or coronally sectioned. Another part was prepared for the cell culture.

3.2.1 Human Sections *(Paper IV)*

The sections were washed in PBS for 30 min and then incubated for 60 min in PBS containing 1% bovine serum albumin (BSA), 0.3% Triton-X and 0.1% sodium azide. Sections were incubated with the following antibodies: rabbit anti-Sox2 (Millipore, 1:200), rabbit anti-Musashi 1 (Millipore,1:200), mouse anti-β-III-tubulin (Millipore, 1:100), mouse anti-NeuN (Millipore, 1:100), mouse anti-GFAP (Chemicon, 1:1000), mouse anti-O4 (Chemicon, 1:50) and rabbit anti-p53 (Santa cruz, 1:400). All sections were incubated with the primary antibody for 24 h at 4°C, rinsed in PBS and subsequently incubated with species-specific secondary antibodies conjugated with Cy3 (goat anti-mouse, donkey anti-mouse, goat ant-rabbit or donkey anti-rabbit)

(Jackson ImmunoResearch) (1:500) and Alexa488 donkey anti-rabbit (1:500) (Invitrogen). Sections were also counterstained with the nuclear marker DAPI (Invitrogen, 1:2000) or TO-PRO-3 (Invitrogen, 1:10000). Sections were mounted in Mowiol (Calbiochem). For negative controls the primary antibody was omitted (data not shown). For the Avidin Biotin Complex (ABC)-technique sections were incubated with biotinylated secondary antibodies (Vector Laboratories) (1:200) for 1 h at room temperature, rinsed in PBS and incubated with ABC (Vectastain ABC Kit) for 1 h. After another step of rinsing in PBS followed by TRIS- hydrogen chloride buffer (0.1 M, pH 7.45) immunoreactivity was revealed by incubation in 3, 39-diaminobenzidine (DAB) by using the DAB Substrate Kit for Peroxidase (Vector Laboratories) for 2–10 min. Sections were rinsed in TRIS and dehydrated through a series of rinses with increasing strength of ethanol solutions to pure xylene and mounted in a non-aqueous DPXmedium.

3.2.2 Human Cell Cultures *(Paper IV)*

Connective tissue was peeled off and the FT was mechanically dissociated with scalpels and scissors and placed in a dissociation medium, consisting of 200 U/ml DNAse (Sigma-Aldrich) and 0,025% trypsin (Invitrogen) or 10 U/ml papain (Worthington) in a 37°C water bath for 30 min. Tissue was triturated three times every 5 min, and further incubated. To stop the enzymatic reaction, 10 mg/ml BSA (Sigma-Aldrich) and 10 mg/ml ovomucoid (Worthington) were mixed with L15, added and mixed with the dissociation medium. Cells were collected by centrifugation at 220 g for 5 min. To further enrich for progenitor cells, 0.9 M sucrose in Hanks' Balanced Salt Solution (HBSS) (Invitrogen) was added to the tissue solution followed by centrifugation at 750 g for 10 min and washing with L15. The cell pellet was re-suspended and to allow single cell cultures in 5 or 10 cm Ø petri dishes in neurosphere medium, composed of DMEM/F12 (invitrogen), HEPES (Gibco), B27 supplement (Gibco) and Penicillin-Streptomycin (Invitrogen). To propagate human FT progenitor cells, the following growth factors were added; 20 ng/ml recombinant human epidermal growths factor (EGF, R&D systems) or mouse EGF (BD Bioscience) and 20 ng/ml recombinant human basic fibroblast growth factor (bFGF, R&D systems). After the first passage, 10 ng/ml recombinant human leukaemia inhibitory factors (LIF) (Chemicon) were added to the medium. The neurospheres were cultured until they reached a critical size (that is not becoming dark/necrotic in the center) which normally took seven weeks. The NPC cultures were propagated and passaged with papain/DNAse twice and used in experiments as single cells after the second passage. Another set of cells was cultured in neurosphere medium with the addition of 20% BIT 9500 medium. BIT medium consists of BSA, insulin (SIGMA) and 20 ng/ml transferrin (Stem Cell Technologies). Platelet-derived growth factor- BB (PDGF-BB) (R&D systems) was added at 30 ng/ml. Cells were incubated for eight days and the medium was changed three times. Cells cultured with BIT 9500 medium were differentiated on gelatine-coated plates (Sigma-Aldrich). In order to exclude contamination of the data by tumor forming cells, all tissue preparations were screened for the presence of the P53 gene. Cell cultures were fixed with 4% PFA and blocked in PBS, 0.1% saponin and 5% goat serum. Preparations were incubated with the primary antibodies; rabbit-

anti-GFAP 1:1000 (DAKO), mouse anti-h-Nestin 1:1000(R&D Systems), mouse anti--b-III-tubulin 1:50 (Chemicon) for 24 h at 4°C, rinsed in PBS and subsequently incubated with species-specific secondary antibodies diluted in PBS; Alexa 488 donkey anti-mouse 1:500 (Molecular Probes/Invitrogen), Cy3 donkey anti-rabbit 1:1000 (Jackson Immunoresearch). Cells were counterstained with nuclear markers Hoechst (Molecular Probes, Invitrogen). The tissue was mounted in Mowiol (Calbiochem, VWR International). In order to evaluate the proliferation capacity of FT another set of culture was performed as previously described. After passage the neurosphers were pulsed with 5-bromo-2'-deoxyuridine (BrdU, Sigma) for 48 h in the presence of EGF and bFGF. The spheres were placed on coated glasses and before fixating them with PFA 4%. The glasses were stained for BrdU, Sox2 and DAPI (data not shown). BrdU incorporation was detected using rat anti-BrdU (1:50, AbD, Serotec) and revealed by secondary antibody goat anti-rat IgG Alexa 488 (1:100, Invitrogen).

3.3 CONFOCAL, FLOURESCENCE AND LIGHT MICROSCOPY

For analysis of the different antibodies a combination of a laser scanning confocal microscope (Leica TCS SPII) (Leica Microsystems) *(Paper III and IV)* and a fluorescence/bright field microscope (Leica DM 400B, Leica DFC320 Leica Microsystems) *(Paper I, II, III and IV)* was used and on which fluorescence and light microscopy was performed. The confocal immunofluorescence images were obtained using a 20x (N/A 0.7) and 63x (N/A 1.40) objective. Alexa 488, Cy3 and Cy5 were excited at 488 nm, 543 nm and 633 nm respectively and detected with a 490–520 nm, 560–630 nm and 640–750 nm band-pass filter respectively. Each optical section (1 μm) was averaged four times; images were the projection of 25 successive optical sections into one image. Images of neurospheres in cell culture *(Paper III and IV)* were taken by using Panasonic CCTV camera (WV-BP312E) (Panasonic) and an Olympus Microscope (CK2 ULWCD 0.3) (Olympus).

3.4 STATISTICAL ANALYSES

For the experiment in *Paper I , II*, and *III* the Mann-Whitney and unpaired two-tailed student t test (GraphPad PrismH 5.0, GraphPad Software Inc,La Jolla, CA, USA) was performed and *P < 0.05 was defined as statistical significance. In *Paper I* the Spearman correlation test was performed. Furthermore Wilcoxon signed ranked test was used and the ANOVA mixed model. The ANOVA mixed model was used because we had two variables, stimuli and time. For further statistical methods used in *Paper II* see previously described statistics in section"1.6.2 Microarray sample preparation and analysis". For colorimetric measurement (*Paper III)* of nitrite normal distribution with confidence interval of 95% was used. In *Paper IV* only unpaired *t* test was used.

3.5 METHODOLOGICAL CONSIDERATIONS

All papers within this thesis include cell culturing and two papers include nitrite measurement (*Paper II and III*). I will here present some methodological consideration concerning these methods.

3.5.1 Cell Culturing

Due to the great difficulties and ethical aspects to obtain human material human NPCs are only used in one study (*Paper IV*) in this thesis. We choose Dark Agouti rat in *Paper II* and *III* since the MOG-EAE model is reproducible, it is predisposing to inflammation in the spinal cord (Storch et al. 1998) and the inflammation in EAE has a similar disease course as in MS patients. Female rats was used which are more prone to be affected by the induction of EAE than their male counterparts. For the FT study (*Paper IV*) male Sprague-Dawley was used due to the size of FT.
In the *in vitro* studies, NPCs were isolated from the diseased CNS and transferred to dishes where the NPCs were manipulated under controlled circumstances. These *in vitro* experiments are important to achieve a greater knowledge about the NPCs intrinsic function and capacity. Another advantage of *in vitro* studies is the reduced amount of experimental animal used when NPCs may be propagated, frozen and be the subject to many different studies.
NPCs in this thesis were obtained from adult animals while in the human study NPCs are as well derived from postnatal and adult donors in order to investigate the age dependent proliferation. Adult NPCs are more fragile and heterogenic than embryonic NPCs or cell-line derived NPCs which often are immortalized. Our aim was to investigate the vulnerability of adult NPCs which is why we do not use cell lines or embryonic NPCs.
Cultured adult NPCs can easily be manipulated by for example environmental factors such as temperature and oxygen, co-culturing with other cell types, genetic manipulation or soluble factors such as $NO^{\cdot}$, cytokines and growth factors (EGF, bFGF). Weiss *et al.* demonstrated that different concentration of EGF/bFGF throughout the neuroaxis were needed for spinal cord NPC culturing (Weiss et al. 1996). In our *in vitro* studies the same concentration of these growth factors are added to NPCs obtained from different part of the spinal cord. Any observed differences in NPCs may of course be a result from a lack of not yet known crucial factors needed by the NPCs, maybe in a region-specific way. The differentiation may also be affected by the type of coating on the plates and the concentration of NPCs seeded. If neurospheres are seeded on coated plates for differentiation the sphere size is critical for the outcome of differentiated cells. We therefore used only single cells for our experiments. In *Paper I* beside EGF and bFGF, heparin was added. FGF has been demonstrated to be highly unstable in 37°C which is the temperature used for NPCs culturing (EGF stability was not affected by this temperature). By adding heparin FGF was stabilized which resulted in an increased proliferation and neurogenesis in embryonic NPC cultures (Caldwell et al. 2004). Heparin has also been proved to interfere with Griess reaction during nitrite measurements and therefore not used in *Paper II* and *III*.

Leukemia inhibitory factor, LIF, has been demonstrated to increase proliferation in adult mouse and human spinal cord derived NPCs (cultured on adherent substrate) but not in adult NPCs obtained from rat dentate gyrus or human embryonic spinal cord (Azari et al. 2005, Dictus et al. 2007, Koechling et al. 2011, Mothe et al. 2011). In this thesis LIF was not used in rat NPCs cultures (*Paper I, II, III* and *IV*) but in NPCs from human (*Paper IV*) because our previous experience in culturing of human NPC was that LIF increased their proliferation (data not shown). BIT medium and PDGF-BB were also added to the human NPCs but not to the rat cultures. BIT medium has previously been successfully used in human NPC cultures for cell expansion and PDGF-BB to induce neuronal differentiation which PDGF is involved in (Johe et al. 1996, Williams et al. 1997, Palmer et al. 2001, Erlandsson et al. 2006).
Both human and rat NPCs were obtained and advantages of using rat derived NPCs rather than human NPCs is that the rats have a less heterogenic genetic background, there are available healthy controls, the NPCs are numerous which increase the survival rate and the turnover rate is much higher than human-obtained NPCs. After passage it can take 7 weeks or more for the human NPCs to form neurospheres.
To obtain living and proliferating cell cultures some procedures of the culturing process can be recommended. The two main issues faced was (I) problem with low cell density and (II) dissociation of cells.
(I) Cells like to be with other cells due to the fact that they produce growth factors (Martino et al. 2006) that is, small culturing plates for small amount of tissue is preferable. For human brain-derived NPCs the critical density seems to be 300 cells/0.33 cm^2 (Arsenijevic et al. 2001) which was a higher density than the FT NPCs cultured in our experiments especially for the rat FT which is extremely small. Neurpspheres formed were few, very small and slowly proliferated. Rat NPCs were over all easier to culture even though within the neuroaxis there were obvious regional discrepancies in proliferation where the FT and the thoracic part in control animal was hardest to culture. This may be due to the low amount of cells or regional differences because despite culturing the thoracic part at the same density as the other parts of the spinal cord (*Paper III*), the cells were slower and harder to keep alive. This resulted in a lower concentration of cells after passages.
(II) Human NPC tissue is rare, precious and fragile. One of the most critical part of handling the NPC was to isolate as high amount of living NPCs from the tissue as possible. FT contains fibrous tissue and requires strong enzymatic treatment during the isolation procedure; this may result in loss of cells or not good enough isolation. The amount of enzyme used is correlated to the amount of tissue and type of tissue. The enzyme papain/trypsin and DNAse is preferably added to the cells at the first step of passage, if the tissue is not totally dissolved it harbors NPCs which are hidden and can be left inaccessible within the culture. At the next passage the tissue may be removed or be dissolved. It may be more cell protective to use other enzymes on the NPCs at the second passage like Accutase®. Accutase® which is a mix of enzymes has worked nicely on human brain derived NPCs which contains less amount of fibrous tissue. Using this enzyme less mechanical dissociation is needed which is also preferable. Sometimes the tissue must be removed during the surgical procedure by using bipolar electrocoagulation which may burn some parts of the tissue piece. This results in fewer viable cells but nevertheless it is preferable to include this tissue part in the cell isolation procedure (we have also tried other enzymes for this kind of FT tissue without any obvious increase in number of NPCs isolated).

The FT cultures were not filtered due to the great loss of tissue and cells. Culturing with conditioned medium or different concentration on growth factors did not increase proliferation. Actually rat NPCs died when doubling the concentration of bFGF. Other important factors for NPC survival were to isolate NPCs immediately after receiving the tissue and add fresh growth factors every second day. All solutions used on the cells should be room temperature. A significant part of the NPCs always attaches to the wall of pipettes and vials which can be avoided through carefully flushing walls of vials and pipettes. The time working with NPCs outside the incubator should be as short as possible. It has also been demonstrated that adult human spinal cord NPCs required an adherent substrate for expansion in culture and be more receptive to LIF (Mothe et al. 2011). This as well as refinement of growth factors, cytokines and other supporting factors may in the future be ways to reach a better outcome. Not forgetting that the NPCs are easily manipulated and adding "factors" may complicate the interpretation of the results.

3.5.2 NO·

NO· is a lipohilic gas which has a short half-life in blood (only a few seconds) and can easily diffuse through cell membranes. Most immune cells have the capacity to produce NO·. Under inflammatory conditions the inducible isoform of its enzyme nitric oxide synthase, iNOS, is activated, which leads to excessive production of NO· (Salter et al. 1991). Increased levels of NO· oxidation products have been demonstrated in EAE (Koprowski et al. 1993) and NO· inhibition also ameliorates EAE (Danilov et al. 2005).

By changes in pH towards a more acidic milieu like the CSF, NO· is stable for a longer period of time. An increase in pH gives an oxidation process forming nitrite and nitrate. These compounds may be measurable with Griess reagent (Griess 1864, Griess 1879). During the Griess reaction nitrate is reduced to nitrite and the method provides the sum of these two compounds. Nitrite reacts during acidic conditions with sulfanilic acid this produces a diazonium salt. Following addition of azo dye the solution develop a pink color which is detectable with an ELISA reader (Tsikas 2007). Another method used for nitrite measurement is capillary electrophoresis which can analyze very small quantities but the samples must be fresh and not previously frozen. Previous studies have demonstrated that this method is more sensitive than applying Griess method when measuring nitrite in CSF from MS patients (Ikeda et al. 1995). We did not successfully found any significant nitrite levels in culture medium obtained from EAE affected rats SVZ. Only spinal cord derived cultures presented detectable levels. This may be due to the model used where the inflammation is primarily localized in the spinal cord (Storch et al. 1998) Applying Griess method on culture supernatants it is of great importance that the cells are seeded at the same density and the amount of medium used is the same. This was implemented in our studies. The Griess reaction measurement can be affected by for example hemoglobin, heparin, heavy metals, high concentrations of sulfate, chloride, and bromide, NADH, thermal instability and turbidity. Particles in the supernatants which cause turbidity scatter light during the measurement. To avoid this samples were filtered prior the analysis. To prevent contamination of nitrite in the vials the sample collection vials were autoclaved and carefully washed in deionized (nitrite-free) distilled water before storage of supernatants. In *Paper III* the focus was to study the low-inflamed areas which were detected by "normal" production of nitrite in cell culture medium. The normal distribution of healthy control nitrite production

was calculated and thereafter a “cut-off” at ≥2SD was decided to be identified as high nitrite production i.e. inflamed parts. Comparing the EAE scores of the animal and nitrite value we found that some critically ill animals had a low nitrite production. This could either be due to the studied spinal cord level did not have a high grade inflammation or that the inflammatory event had ceased. Since $NO^{\cdot}$ formation has been shown to correlate to active inflammation we believe that this is also the case in EAE. However the EAE score did not correlate to nitrite levels it is assumed that the score is composed of both active inflammation and remaining residual functional loss.

4 RESULTS AND COMMENTS

4.1 PAPER I. TLR ACTIVATION INDUCES TNF-A PRODUCTION FROM ADULT NEURAL STEM/PROGENITOR CELLS

In this study we investigated: I) If NPCs express TLR2 and TLR4 during normal physiological conditions. II) If different pro-inflammatory cytokines modulate the expression of TLR receptors in NPCs. III) If the TLR receptors on NPCs can be activated and induce cytokine release.

4.1.1 Neural Progenitor Cells Expressed Toll Like Receptor 2 and 4 Constitutively

Flow cytometry and immunohistochemical analyses were performed to determine if NPC express TLR2 and TLR4 receptors. We demonstrated that these receptors were expressed *in vitro* during normal physiological conditions. Furthermore, Sox2 - expressing cells *in vitro* were positive for TLR which indicated that the NPC were undifferentiated and the expression of the TLRs was not due to differentiation into an already known TLR-expressing cell. 99% of the Sox2 positive cells expressed TLR4 which revealed a homogenous expression. Immunohistochemistry on brain sections from adult rats revealed TLR2 and TLR4 expression within the SVZ.

4.1.2 Inflammatory Stimuli Up-regulate Toll Like Receptor 2 and 4

When NPC were exposed to pro-inflammatory conditions the cells upregulate TLR receptor expression. In order to mimic an inflammatory environment, supernatants from activated macrophage cultures were added to the NPC cultures which induced TLR2 expression. In turn the TLR2 expression in the NPCs correlated to the TNF-α levels in the added macrophage supernatant. The positive correlation was significant. In a second step, TNF-α and IFN-γ was added to the NPC cultures. Both cytokines

induced TLR2 expression both single-handedly and synergistically. However, only IFN-γ was able to induce TLR4 expression and this expression was abrogated by TNF-α.

Since presence of microglia, CD11b positive cells, would affect our result we needed to quantify the number of microglia and CD11b immunolabeling of the cell cultures was performed. The result showed that cultures contained 0.1-0.7% CD11b positive cells. TLR expression was therefore measured in sorted, CD11b depleted cultures after exposure to TNF-α and IFN-γ. Also after sorting TLR expression revealed the same pattern as the non-sorted NPC cultures which demonstrates that the presence of microglia was not the cause of the elevated TLR expression but rather originated from TLR upregulation on the NPCs.

TLR activation did not induce changes in NPC proliferation or neuronal/astroglial differentiation.

4.1.3 Toll Like Receptor 2 and 4 Agonists Induce TNF-α Protein Production in the Neural Progenitor Cells

The TLR agonists LTA, Pam3Cys, LPS, and HMGB1 were added to the cultures in order to determine the role of TLR2 and TLR4 in NPCs. LTA engage the heterodimer TLR2/TLR6, Pam3Cys (trihydrochloride, a synthetic analog of the naturally occurring lipoprotein) ligates the TLR2/TLR1 heterodimer, whereas LPS binds to TLR4. HMGB1 binds to both TLR2 and TLR4. Using FACS staining it was evident that both stimulated with Pam3Cys and unstimulated NPC cultures stored intracellular TNF-α. RT-PCR results revealed that Pam3Cys induced TLR2 expression. None of the TLR agonists induced TLR4 but all of them induced TNFα mRNA where the agonist Pam3Cys induced TNFα in a statistically significant manner. TLR2 activation stimulated the release of TNF-α from the NPCs which was measured in the supernatant using ELISA quantification.

In conclusion; TLR2 and TLR4 are expressed *in vivo* and *in vitro* in NPCs. The expression of these receptors was transcriptionally upregulated after inflammatory stimuli. The expression of TLR2 and TLR4 was differently affected by two major proinflammatory cytokines evident in EAE, MS and trauma. Activation of TLR2 and TLR4 receptors on NPCs resulted in the synthesis of TNF-α which was released from the NPCs in the supernatant.

4.2 PAPER II. CHANGE OF FATE COMMITMENT IN ADULT NEURAL PROGENITOR CELLS SUBJECTED TO CHRONIC INFLAMMATION

NPCs were isolated from SVZ and spinal cord of MOG-immunized EAE rats as well as from non-immunized controls to investigate if there were any transcriptional or functional regional differences. NPC were isolated from SVZ, cervical, thoracic and caudal part of the spinal cord. The Global transcriptome was measured by using Affymetrix GeneChip® Rat Gene 1.0 ST arrays in undifferentiated and differentiated cultures. The gene expression analysis was paralleled by experiments on functional differentiation of NPCs.

4.2.1 Functional and Gene Expression Analyses Reveal Regional Differences in Neural Progenitor Cells

In *undifferentiated* NPCs we detected a significant ($p<0.001$) difference in gene expression of 187 genes between the SVZ and spinal cord NPCs. The SVZ derived NPCs had a higher gene expression in 183 of the 187 differently expressed genes compared to the NPCs derived from the spinal cord. Four genes had a higher expression in spinal cord-obtained NPCs. We identified this by using the statistical analysis one-way ANOVA with adjusted Bonferroni correction for multiple testing and Dunn's post test. The same statistical procedure was performed on gene expression data from *differentiated* NPCs. In SVZ-derived NPCs the expression of 146 genes had a higher expression than NPCs from the spinal cord. To functionally classify the differentially expressed genes we used the WEBGESTALT platform. We could detect (both in genes from undifferentiated and differentiated NPCs) a cluster of neuronal differentiation-related genes. In the undifferentiated NPC gene pool, these genes were: *Lkh2, Notch3, Ptk2, Cxcr4, Rorb, Emx2, Etu/Pea3* while among genes from differentiated NPCs the neuronal cluster was composed of: *Dcx, Map2, FoxG1, Lhx2, Nnat* and *Pou3f2.*

Differences in gene expression were also reflected in the functional outcome of differentiated cells. When differentiated NPC cultures from the four CNS segments were histo-chemically immunelabeled and quantified, SVZ-derived NPCs generated significantly more β-III tubulin during healthy conditions than cells than NPCs from

spinal cord which generated significantly more oligodendrocytes. From these results we could conclude that SVZ-derived NPCs display a stronger neurogenic fate than their spinal cord counterparts in normal conditions.

4.2.2 Inflammation Affects the Caudal Neural Progenitor Cells

When compared control to EAE affected animals significant gene expression changes (FDR $\leq$ 0.05 and -1.2 $\geq$ fold change $\geq$ 1.2) were detected in the caudal undifferentiated NPC group. Using the Multi Experiment Viewer (MeV) platform we detected two major gene clusters of co-regulated genes. The gene expression was functionally classified using the WEBGESTALT platform. With this tool we could identify that a cluster with higher expression in EAE was enriched in immune-related genes (as expected) whereas genes with higher expression in control NPCs were enriched in developmental process-related genes. Ingenuity System Pathway Analysis (IPA) was performed on the entire data set. IPA confirmed the results from the WEBGESTALT analysis. In the spinal cord NPCs a subset of the most decreased functions were related to cell viability, branching/neuritogenesis and lipid metabolism while the most increased functions involved neurodegeneration and inflammation. In neuroinflammation the analysis on nervous system-related genes demonstrated that the most significant changes involved downregulation glial-related functions such as myelination, quantity of Schwann cells, survival and morphology of oligodendrocytes, quantity and proliferation of neuroglia. Within these datasets we could detect genes with pivotal function in astrogliogenesis (*Cntf, Stat3, Fgf3 and Shh)* oligodendrogenesis (*Shh, Nkx6-2, Erbb3, Fgf2)* and oligodendrocyte differentiation (*Thra, Lingo1, Rtn4, p73 and Aspa).* Several canonical pathways involved in gliogenesis were down-regulated: CNTF, IGF1, FGF, JAK/STAT. Taken together the arry data suggested that inflammation decreased the astro and oligodendroglial potential of spinal cord derived NPCs and induces gene changes involved in neurodegeneration.

4.2.3 Inflammation Skews the Differentiation fate of Spinal Cord Neural Progenitor Cells from Gliogenic to Neurogenic

Immunocytochemistry and western blotting for β-III tubulin, GFAP and GalC on differentiated cultures was preformed. Oligodendrocyte differentiation was unchanged in EAE-derived SVZ, whereas in spinal cord NPCs oligodendrocyte differentiation was significantly decreased compared to normal controls. This was confirmed with western blotting for GalC. Astrogliogenesis was significantly increased in SVZ derived EAE but in the spinal cord the situation was reversed which also was confirmed by in the western blot for GFAP. In EAE the neuronal differentiation was increased in the spinal cord as measured by immunohistocemistry and western blot. In this model neurogenesis in SVZ NPCs was unaffected by EAE.
Gene expression in NPCs was also studied with RT-PCR at 0h, 24h and 5-7 days post-differentiation. The *Notch1* expression was increased in EAE derived spinal cord NPCs at 24hrs. *Hes1* was instead down-regulated 0h in cervical part. *Mash1* expression had a tendency towards an increase in spinal cord NPCs at all time-points. *β-III-tubulin* in the spinal cord was increased at later time-points 5-7 days which was in line with the finding of increased neurogenesis.

In all, this study reveals a regional difference in NPCs neurogenic and gliogenic potential in the healthy situation but also how that inflammation skews the fate of the spinal cord NPCs towards neurogenicity.

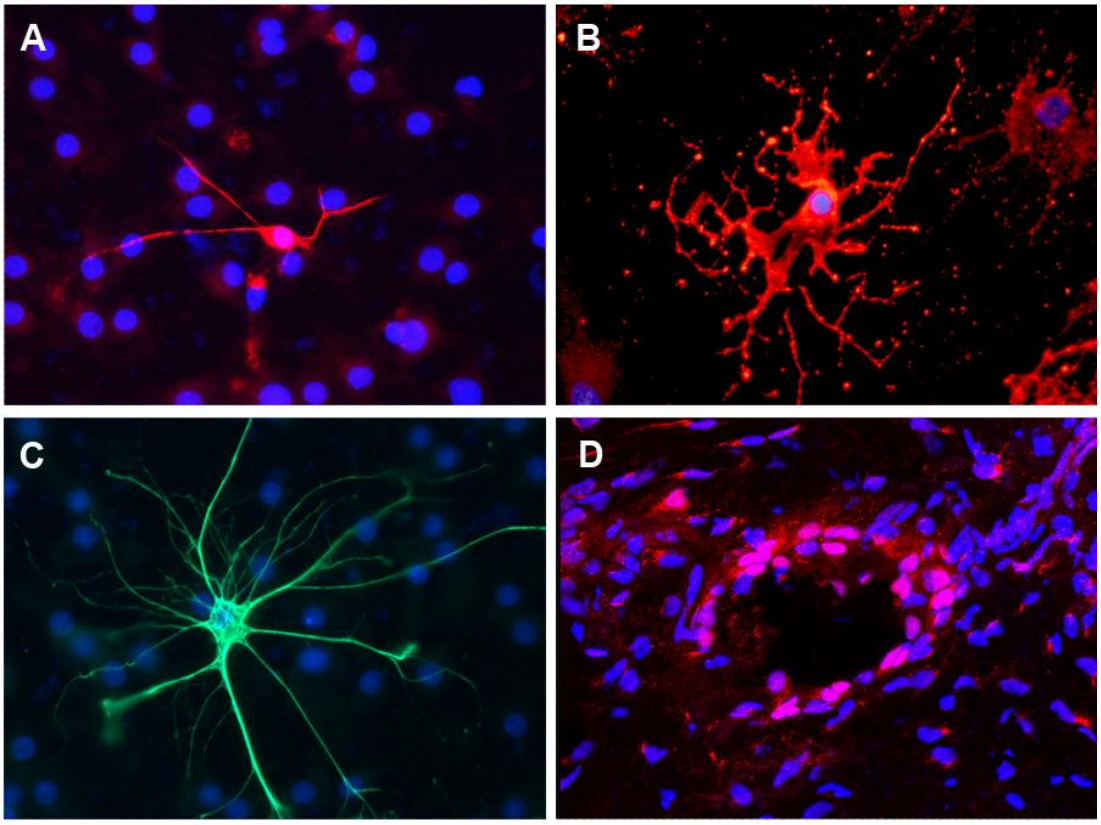

Figure 1. Adult rat spinal cord NPCs, can turn into three linages; neurons, astrocytes and oligodendrocytes. Differentiated cells immunolabelled with A) β-III tubulin for neurons (red), B) oligodendrocyte is stained for GalC (red) and C) GFAP for astrocytes (green). DAPI was used as nuclear marker (blue). D) Human filum terminale, FT, harbors neural progenitor cells, NPCs, *in vivo* which are immunolabeled with NPC marker Sox2 (red) and nuclei labeled with TO-PRO-3 (blue).

4.3 PAPER III. ALTERED GENE EXPRESSION AND DIFFERENTIATION IN SPINAL CORD NEURAL PROGENITOR CELLS AFTER EXPOSURE TO LOW LEVEL INFLAMMATION

The previous study (*Paper II*) revealed a significant difference in features of SVZ and spinal cord-derived NPCs during normal physiological conditions and an alteration of differentiation fate in the spinal cord NPCs during inflammation. In this study we focused on NPCs from levels within the EAE affected spinal cord which did not show signs of high level inflammation. The purpose of this study was to evaluate to what extent NPCs were affected by distal or non acute inflammation.

4.3.1 Nitrite Production as a Marker for Ongoing Inflammation

The spinal cord was divided, isolated and cultured the same way as in *Paper II*. Previous studies have shown increased levels of $NO^{\bullet}$ oxidation products in EAE (Koprowski et al. 1993) and that $NO^{\bullet}$ inhibition ameliorates EAE (Danilov et al. 2005). In cell culture medium $NO^{\bullet}$ is oxidized to nitrite and nitrate. To investigate the level of ongoing inflammation the nitrite levels in NPC cultures supernatants from EAE affected animals were measured by using the Griess reaction (Griess 1864). The normal distribution of nitrite in NPC culture supernatants from 24 non-immunized control rats was used to determine the normal range of nitrite levels found in healthy conditions. Ultimately, this value was used to determine the cut-off between "normal" levels of nitrite from healthy animals and inflammatory nitrite levels from EAE animals. Nitrite levels ≥+2SD from the mean of normal were considered to constitute ongoing tissue inflammation. Spinal cord segments derived from EAE affected rats which had a nitrite levels below the decided cut off (mean +2SD of the healthy control animals normal distribution) were defined as "Normal Appearing Spinal Cord", NASC. Segments with nitrite levels above or equal to the mean + 2SD of that of controls were defined as "Inflamed Spinal Cord", ISC. 28 EAE affected rats were studied. Fourteen cervical, 16 thoracic and 18 caudal parts had normal nitrite levels and were defined as NASC.

4.3.2 Inflammation Increased Proliferation in Spinal Cord Thoracic Neural Progenitor Cells

During culture conditions cells were cultured at similar density. In cultures from non-immunized controls NPCs from the thoracic part generated significantly lower numbers of NPCs compared to the other spinal cord parts. On the other hand the thoracic NPCs responded to the inflammation by massive proliferation. Using [^{3}H]thymidine incorporation a significantly higher proliferation of NPCs (14 times higher) was detected in thoracic derived cultures as compared to control NPC cultures. To ensure that proliferation was caused by NPCs (and not other cells) we performed double-labeling with BrdU and Sox2 which confirmed that the Sox2 positive cells were the proliferating cell pool. A CD11b/BrdU double staining was also done to determine the

extent of microglia involvement in the proliferation. The ratio microglia/BrdU positive cells was 1/500.
When NPCs derived from cervical and thoracic NASC segments were differentiated the procedure revealed a significant decrease in the number of oligodendrocytes compared to controls. On the other hand β-III-tubulin labeling of NPCs showed overall increase in neuronal numbers which was significant in the thoracic part. No differences were found in cellcounts from GFAP stainings.

4.3.3 Gene Expression and Differentiation in Neural Progenitor Cells was Affected at a Distance from Inflammation

Using RT-PCR, the expression for "stemness" genes and differentiation-related genes were studied in NPCs isolated from different levels of the spinal cord. The expression was measured at different time points after induction of differentiation: at 0h in undifferentiated NPCs and at 24h and 5d in differentiated NPCs. *Notch-1* was significantly increased in NASC in the caudal part and in pooled NASC parts in undifferentiated (at 0h) and in the differentiated (24h, 5d) state compared to normal controls. *Hes-1* displayed a significant decrease in gene expression in undifferentiated NPCs from NASC. *Mash-1*, *Neurogenin2* and *β-III-tubulin* gene expressions were upregulated in NASC compared to control in differentiated NPCs. Gene expression of *Gfap* was upregulated in the caudal part of the spinal cord in NASC areas. Gene expression data was also pooled from different spinal cord segments acquired at the same time-points. *Notch, Hes-1 and Mash-1* were altered at early time points where *Notch* and *Mash-1* expression was increased, in contrast *Hes-1* was significantly decreased. The *β-III-tubulin* gene expression was significantly upregulated in NASC-derived NPCs at 24h and 5 days when all spinal cord segments were pooled. The gene expression analysis revealed an altered expression in areas with low levels of ongoing inflammation (NASC). Taken together the NPCs in NASC areas had a gene expression pattern suggesting to promote neurogenesis. Increased neurogenesis was also found in the functional analysis of cell counting of β-III-tubulin expressing cells.
A bioassay study was preformed to investigate if soluble factors from inflamed tissue released in the medium *in vitro* could divert the fate of normal NPCs or account for the increased proliferation. Control NPCs were cultured with conditioned medium from EAE-inflamed cultures. Conditioned medium from NPC from non-immunized rats was

used as control. Neither proliferation (assessed by [^{3}H]thymidine incorporation) nor differentiation (Gal C, β-III-tubulin and GFAP stainings) was affected by the EAE conditioned medium. An interpretation of this result is that the priming changes detected occurred under fate commitment in NPCs from EAE animals was a result of the *in vivo* exposure to the inflamed environment.
In summary, spinal cord derived NPCs were affected by a distant inflammation in aspect of proliferation, differentiation and gene expression.

4.4 PAPER VI. DISTRIBUTION AND CHARACTERIZATION OF PROGENITOR CELLS WITHIN THE HUMAN FILUM TERMINALE

In this study we wanted to investigate if the most distal part of the conus medullaris, the filum terminale contained neural precursor cells and if these cells could be expanded and differentiated. FT tissue was obtained from patients (1-60 years) undergoing surgery for tethered cord. Half through the study Varghese *et al.* published a paper where they described that indeed this was the case (Varghese et al. 2010). We then needed to expand our paper, use additional immunelabeling and add information on distribution of NPCs within the tissue. Recently a third paper which confirmed this finding was published (Jha et al. 2012).

4.4.1 Filum Terminale Derived Neural Progenitor Cells Proliferate and Differentiate *in vitro*

NPCs from human FT tissue were isolated and propagated in stem cell culture medium. Single cells were able to form neurospheres in 13 out of 21 patients (62%). The spheres resembled spheres derived from SVZ and spinal cord NPCs although we did not quantify proliferation by sphere assay. FT NPCs obtained from tissue isolated from younger donors seems to have a higher growth- and proliferation capacity and could be passaged up to 15-30 passanges (n=6). NPCs from older donors could be propagated but could be passaged a significantly lower number of times compared to NPCs from younger donors. Using double labeling with Sox2/BrdU proliferation of the spheres we could determine that the NPC population was the source of proliferation.

In early stage of differentiation Nestin positive immunohistochemically-labeled cells were visualized. GFAP-positive cells with phenotypic and morphological characteristics of astocytes were present after 11 days of differentiation. The neuronal marker β-III-tubulin was positive in 5% of the differentiated cells. After the NPC cultures have been passaged 29 times the cultures were differentiated and β-III-tubulin positive cells could still be detected. After exposing the NPC cultures for the mitogen PDGF-BB, the cells which expressed the neuronal labeling β-III-tubulin, was significantly increased compared to control cultures. Differentiated NPCs labeled positive for oligodendrocyte markers were not found.
In summary, it was possible to isolate and propagate NPCs obtained from FT. The neurospheres obtained were similar to neurospheres derived from SVZ and spinal cords NPCs and the cells responded to growth factors in the same manner. NPCs derived from younger patients had a higher proliferative ability but NPCs from older donors could still be propagated. These results suggest that FT contain a NPC pool which is maintained in adulthood.

4.4.2 Filum Terminale Harbors Neural Progenitor Cells *in vivo*

Immunohistochemistry was used to investigate the distribution of NPCs *in vivo*. Sagittal and coronal sections were labeled with NPC markers Sox2 and Musashi-1. Both Sox2 and Musashi-1 positive cells were visualized in the FT. Sox2 labeled cells appeared in subependymal bands, small and large clusters with up to 500 cells/sectioned cluster and Sox2 positive cells were surrounding the central canal. Sox2 positive cell clusters were found throughout the tissue and no rostro-caudal gradient was detected. Musashi-1 was also found in the ependymal and subependymal area around the central canal as well as in smaller islets at a distance to the central canal. Double labeling revealed that Musahi-1 immunoreactivity coincides with Sox2 immunoreactivity. Furthermore smaller clusters of Sox2 positive cells were GFAP positive. These cells revealed a strong GFAP and Sox2 immunoreactivity. Cells with a weaker GFAP immunoreactivity were found in the surrounding tissue.
We also made a comparison between rat and human FT. Longitudinal sections from both species confirmed the presence of a long central canal structure with a border of ependymal cells. The FT also contained vascular, fibrous and fatty tissue as well as collagen bands. In the rat, the central canal was larger and more defined than the human

central canal which was found to be a more scattered system of tubular structures. In the rat central canal the structure containing ependymal cells was found at the conus medullaris level and was more prominent at a caudal level. Rat FT expressed nestin *in vivo*. *In vitro* neurospheres were obtained from 5 out of 13 cultures (39%).
Finally, NPCs were found to a high extent throughout the FT *in vivo* in both human and rat. Some of the Sox2 positive NPCs were also positive for GFAP which is similar to NPCs labeled in SVZ and spinal cord. FT may be a source for autologous transplantation of NPC in the future.

5 DISCUSSION

5.1 TOLL LIKE RECEPTOR EXPRESSION IN NEURAL PROGENITOR CELLS (*PAPER I*)

The gene *Toll* was discovered in *Drosophila melanogaster* to be involved in dorsal-ventral patterning and synaptogenesis during development (Anderson et al. 1985). In 1996 Lemaitre *et al*. demonstrated the Toll receptor to be involved in the immune response in adult *Drosophila* (Lemaitre et al. 1996) and in 1998 Poltorak and Beutler described the immune function of *tlr4* in mice (Poltorak et al 1998). TLRs are involved in CNS inflammation (Ransohoff et al. 2012) which occurs during most CNS pathogen such as trauma, stroke, cns-infections as well as in autoimmune and degenerative diseases.

Proliferation and differentiation of NPCs are affected by various cytokines (see table 1). It has previously been shown that NPCs express the co-stimulatory molecules B7-1 and B7-2 (Imitola et al. 2004) and furthermore upregulate TLR2 and TLR4 receptors in neuroinflammatory areas in MS and EAE (Andersson et al. 2008). For these reasons we became interested in the TLR2 and TLR4 receptors and the innate immunity connection with NPCs.

In our study we found TLR2 and TLR4 expressing NPCs as well *in vivo* as *in vitro*. Furthermore activation of these receptors induced NPCs to synthesize TNFα which was released in the supernatants. In comparison to activated macrophages the stimulated NPCs could generate 1/5 of macrophage TNFα production which was surprisingly high for a non-immune cell. It may have an impact on the sensitive NPC niche. Since TNFα induced TLR2 expression this may indicate that an autocrine inflammatory loop is created in which inflammation may establish a chronic inflammatory state. The overall TNFα effect on adult NPCs is not totally coherent (see table 1) and it is of value to investigate how NPCs are reacting following a prolonged TNFα exposure. It may even turn out to that TNFα have supporting effects on NPCs for example increased proliferation and neurogenesis (Wu et al. 2000, Heldmann et al. 2005, Widera et al.

2006). Furthermore TNFα has two receptors and the effect on one or the other of these on NPCs or other cells are not to date fully established.

Previous studies has shown that TLR receptors increase neurogenic differentiation of NPCs in SGZ (Rolls et al. 2007). We did not detect any change in gene expression or functional differentiation of *gfap* and *β-III-tubulin* following TLR2 and TLR4 agonist stimulation of the NPCs. This difference may be due to differences in region of origin and species from where NPCs were obtained. We added IFNγ to the agonists which increased the expression of *β-III-tubulin* this has also been demonstrated by Zahir et al who described the same effect on SVZ NPCs (Zahir et al. 2009). In *Paper II* and *III* we found that inflammation did change differentiation fate of the NPCs. In *Paper II* and *III* we suggest that the inflammatory action on the NPCs occurs *in vivo* and the affect on NPCs may later be observed *in vitro.* Since direct stimulation of TLR receptors did not change NPC fate we conclude that this fate changes are not mediated via TLR signaling.

TLR2 and 4 are involved in a variety of pathological conditions as a fist line of defense against pathogens. In pneumococcal CNS infection TLR2 and 4 signaling have been demonstrated to be important for recovery (Klein et al. 2008) as well as for viral meningitis where TLR 2 is important for viral protection in the individual (Sorensen et al. 2008). Kigerl *et al.* published that TLR2 and 4 increased following spinal cord injury and absence of these receptors in TLR4 mutant and TLR2 knockout mice impaired the motor function recovery (Kigerl et al. 2007, Kigerl et al. 2009) whereas in brain trauma the activation of these receptors are linked to a poor outcome (Park et al. 2008, Sansing et al. 2011). By intraspinal injection of TLR4 agonist Schonberg *et al.* detected an increase in oligodendrogenesis but that TLR2 agonist injection mediated demyelination (Schonberg et al. 2007). TLR4 has also been associated with pain in spinal cord injuries (Clark et al. 2010).

Attempts to block the TLR receptors as a therapeutic tool for CNS trauma or inflammation may however be deleterious for the patient due the more or less complete inhibition of the innate immune system.

In conclusion, the three main findings in this paper were I) NPCs harbors TLR2 and 4 receptors *in vitro* and *in vivo* during normal conditions. II) These receptors could be activated by inflammatory stimuli and III) following this activation NPCs were capable to produce the TNFα protein, at measurable protein levels for extended periods of time.

5.2 EFFECT OF CHRONIC INFLAMMATION ON NEURAL PROGENITOR CELLS *(PAPER II)*

In this project NPCs were isolated from healthy control rats and rats subjected to 30-40 days of chronic inflammation (MOG EAE). To investigate if NPCs from various parts of the CNS were different, we divided the CNS into four segments and analyzed the NPCs separately: 1) SVZ and 2) cervical, 3) thoracic and 4) caudal part of the spinal cord. This analysis revealed interesting differences in NPCs along the rostro-caudal axis both in the healthy animal and after inflammatory disease.

In control animals we found that undifferentiated and differentiated NPCs from the SVZ had a more neuronal potential than spinal cord-obtained NPCs. The neuronal potential in SVZ may mirror the *in vivo* situation where neurogenesis takes part in the brain (Lois et al. 1994, Gage 2000) but not in spinal cord during normal conditions (Meletis et al. 2008). Gene clustering analysis and gene enrichment analysis was performed on undifferentiated and differentiated NPCs which demonstrated that SVZ obtained NPCs had a significantly higher expression of genes involved with neuron development and differentiation. This neuronal potential was also found in functional data when NPCs were allowed to differentiate. In healthy animals the spinal cord-obtained NPCs were instead prone to differentiate into the glial linage. The regional specificity within the healthy CNS has also been demonstrated by other groups: Kulbanski *et al.* which observed that NPCs from the spinal cord cervical part differentiated in the same pattern as the SVZ-derived NPCs but differed from caudally-obtained NPCs (Kulbatski et al. 2009). Shihabuddin *et al.* demonstrated differences in differentiation within NPCs from different levels of spinal cord. They divided the spinal cord in four parts and found increase in glial cells caudally and less neurons thoracally (Shihabuddin et al. 1997). Since we did not divide the spinal cord in this way it is difficult to compare results with ours but nevertheless they found a region-specific difference within the spinal cord. Pfenninger *et al.* presented different gene expression in NPCs from lateral ventricular wall compared to spinal cord-derived NPCs. They found that the majority of genes with a higher transcript levels in spinal cord NPCs than in SVZ-derived cells were involved with cell division, cell cycle regulation, telomere stability and maintenance of dividing cells. Also RA target genes and genes from the *Hox* gene family were found to have a higher expression level in the spinal cord (Pfenninger et al. 2011). This results may suggest that spinal cord NPCs posses the ability for proliferation upon stimuli and with the involvement by genes from the *Hox*

family and that this may be regionally restricted. This up-regulation of genes in spinal cord may partly be the cause of the detected NPC proliferation in the thoracic level after inflammation (*Paper III*). Pfenninger *et al.* also presented that NPCs from lateral wall of the ventricles in the brain displayed a set of gene expression which may explain the NPCs ability to turn into neuroblasts. We also detected up-regulated genes in SVZ which were related with the neuronal linage.

In the EAE model used in this thesis the inflammation is mainly localized to the spinal cord where we also detected the largest significant changes in transcriptional profile after the inflammation. The changes found in the chronic inflammation in undifferentiated spinal cord NPCs were translated into functional fate changes in differentiated cells. After inflammation the spinal cord NPCs had a decreased oligo- and astrogliogenesis and increased neurogenesis. The attempt of neurogenesis in the spinal cord was previously described in the same model (Danilov et al. 2006). It is however uncertain to which extend the immature neuron would survive and integrate to restore function. Also since we found decreased oligodendrogenesis, the newly born neurons may suffer from this decreased oligodendrogenesis.

Other groups have been interested in the effects of inflammation on NPCs and addressed this in animal studies. Pluchino *et al.* have previously presented that chronic inflammation can affect NPCs in the SVZ by impaired proliferation and neuroblast migration (Pluchino et al. 2008) . Tepavcevic *et al* found an increased gliogenic fate in SVZ-derived NPCs after inflammation (Tepavcevic et al. 2011). They created a model where EAE was "boosted" by additional LPS injection close to the regenerative SVZ zone i.e. "targeted EAE". This model increased the level of overall inflammatory load in vicinity to the SVZ NPCs. In their model neurogenesis was decreased and oligodendrogenesis increased. It is interesting to find that the situation was completely reversed in the spinal cord NPCs as presented in our study. Furthermore it is also described in MS that NPC in the SVZ turn into a more gliogenic fate in SVZ which is coherent with our findings in EAE (Nait-Oumesmar et al. 2007, Tepavcevic et al. 2011).

In our studies the detected decrease in spinal cord NPCs ability to different into oligodendrocytes was also found in the array analysis where several canonical pathways involved in gliogenesis such as oligodendrocyte differentiation and lipid membrane metabolism were down-regulated. We also found significant up-regulation of genes associated with neurodegeneration. It has recently been shown by Laule *et al.* that combination of loss of myelin lipids and neurodegeneration was observed in

diffusely abnormal white matter in MS (Laule et al. 2013). Our material might be important for understanding the connection between neurodegeneration and neuroinflammation and further analysis focusing on this issue will be performed on this material.

There are findings which suggest that the inflammatory response is regionally different within CNS. Stromnes *et al* presented that there is a regional specificity regarding IFNγ signaling in EAE inflammation between brain and spinal cord (Stromnes et al. 2008). In trauma Schnell *et al.* investigated the inflammatory response between brain and spinal cord after an incision wound and a stronger acute inflammatory response was found in spinal cord than in brain. In the spinal cord the breakdown of the blood brain barrier was larger and the inflammatory response was distributed over a comparatively larger area (Schnell et al. 1999). Differences in regional inflammation response may also affect the NPCs.

In spinal cord the NPCs are mostly situated around the central canal and compared to SVZ at a lower density. To be able to isolate as many NPCs as possible we used the whole segment (cervical, thoracic or caudal part) of the spinal cord. In the white matter there are precursor cells with a restricted fate (NG2 positive cells, radial glia, and pericytes) present with stem cell properties but most of them do not differentiate into neurons, oligodendrocytes and astrocytes or have the capacity of extended proliferation. It is however likely that these precursors were present in the culture.

In summary, this study gives insight into how NPCs differ in neurogenic and gliogenic potential depending on their origin in the healthy situation. We also demonstrate how chronic CNS inflammation can alter the fate of progenitor cells. This finding may be of important for the understanding of how inflammation alters NPC fate and their regenerative potential.

5.3 NEURAL PROGENITOR CELLS ARE AFFECTED BY DISTANT INFLAMMATION *(PAPER III)*

In *Paper II* we used all parts of the inflamed spinal cord without sorting the segments in relation to the level of on-going inflammation. We found differences between the different CNS segments and further wanted to investigate the NPCs from CNS areas where inflammation was low and compare these NPCs with NPCs derived from normal areas.

To identify the focus of the inflammation the nitrite level (a NO· derivate) was measured in the NPC culture supernatants. Increased levels of NO· has been found in many inflammatory conditions and is suggested to be a reporter of an on-going inflammation (Moncada et al. 1995). In NPCs from low-inflammatory segments (NASC) of the spinal cord we found an increase in neurogenesis and a decreased differentiation into oligodendrocytes which was similar to the NPC cultures analyzed in *Paper II* where NPCs from all segments were used. These results demonstrate that the NASC/ISC separation or identification of the inflammatory focus was not entirely necessary due to the fact that all segments revealed to be affected. This indicates that a more wide-spread part of the CNS is actually affected than just the lesion area. We performed a bio-assay study to determine if the effect was due to the *in vitro* culturing and also if the effect was mediated by soluble factors. The results from the bio-assay study were negative and this may suggest that the inflammatory effect on NPCs derived from a NASC segment was obtained *in vivo*. The duration of inflammation in our rat model is quite short compared to chronic inflammation in patients. The result from analyzing NPC gene and protein expression may just reflect the initial changes. Further studies are needed to investigate if NPCs are affected of this distance effect in spinal cord injury and also how inflammation relates to neurodegeneration.

One weak point with this *in vitro* analysis is that even though there were low levels of nitrite in the medium, the inflammation could have been intense at an earlier time point and subsided at the time of measuring the nitrite levels.

We demonstrated an increased capacity of proliferation during inflammation in NPCs obtained from thoracic spinal cord. It was previously demonstrated that NPCs increase in proliferation *in vivo* and *in vitro* after spinal cord injury (Johansson et al. 1999, Moreno-Manzano et al. 2009, Barnabe-Heider et al. 2010) and *in vivo* after root avulsion (Fagerlund et al. 2011). In the NPC cell cultures from inflamed spinal cord in our experiment the NPC proliferation increased 14 times. Proliferation was also detected *in vitro* after ischemic brain injury and excitotoxic lesion (Moreno-Manzano et al. 2009, Deierborg et al. 2010). We found significantly increased proliferation of thoracic NPCs after inflammation but not in the cervical or caudal obtained NPCs which is not coherent with other studies. Anatomically the lumen of the central canal in rat at thoracic level is lined with fewer ependymal cells than cervical, caudal part and even FT (Bruni et al. 1987). Thoracic NPCs are maybe more scarce but may compensate by increased proliferation during pathological conditions.

Our study presented that *Notch-1* expression was increased both in NPCs at undifferentiated and differentiated state. *Notch-1* expression which is active during development and involved in cell proliferation may support the proliferation found throughout the spinal cord (Artavanis-Tsakonas et al. 1999, Akai et al. 2005).
In all, this study demonstrated that in the inflamed spinal cord the NPCs proliferation and differentiation was independent of the level of active inflammation. Also extensive proliferation was found in the thoracic spinal cord.

5.4 HUMAN FILUM TERMINALE HARBORS NEURAL STEM CELLS *(PAPER IV)*

A reason for studying the FT is that CNS of adult salamanders, fish and reptiles display a high capacity of regenerative ability. The regenerating cells are GFAP positive radial glia-like cells (ependymoglia) (Echeverri et al. 2002, Pinto et al. 2007). The probability for finding remnants of regenerative cells in this area seemed likely, which was the rational for the present study.
FT is mainly clinically significant in the tethered cord syndrome where the patients are treated with surgical division of FT. During this procedure a short segment of FT is usually be removed in order to avoid reattachment of the divided structure. In 2010 while this study was ongoing Varghese *et al.* demonstrated in four patients that FT contains cells with NPC properties (Varghese et al. 2010). This urged us to extend our material and include information on the distribution of the NPCs.
By differentiating the NPCs obtained from the FT *in vitro* we found cells positive for GFAP and β-III-tubulin. No oligodendrocytes were detected after differentiation of FT NPCs. In the rat spinal cord NPCs cultures the myelin is osmotically removed by using a sucrose solution. This procedure was tested on human FT but with a very low myelin yield indicating low density of myelin. This is in agreement with the finding that nerves in the FT are unmyelinated. We may not have used optimal concentrations of for example thyroid hormone, progesterone, heparin or transferrin which are known to promote oligodendrocyte differentiation (Espinosa-Jeffrey et al. 2009, Monaco et al. 2012). Unknown necessary factors may exist since many groups have problems to culture human oligodendrocytes.
Our lab has cultured human NPCs since over ten years. Johansson *et al.* was among the first who successfully isolated NPCs from human SVZ and SGZ and differentiated the

NPCs into oligodendrocytes, astrocytes and neurons (Johansson et al. 1999). Members of our team were also able to harvest cells from SVZ during an endoscopic neurosurgical procedure. These papers characterize the maturation of progenitors, their ability to form synapses and release excitatory and inhibitory transmitters (Moe et al. 2005, Westerlund et al. 2005).

Our proliferation data demonstrated a significant decrease in numbers of cell cycles *in vitro* in NPC cultures obtained from older donors than NPCs from younger donors. This finding could be due to age-related depletion of NPCs in adult SGZ and decreased SVZ neurogenesis in older individuals (Encinas et al. 2011, Wang et al. 2011). Though, regardless of age the NPC cultures had the capacity to proliferate *in vitro* and demonstrated a strong capacity of self-renewal in the clonal expansion study. The NPCs also harbor the property to respond to the growth factor PDGF-BB with significant increase in neuronal differentiation. This capacity is of importance for example for initiating neuronal differentiation before transplantations. Concerning transplantation and PDGF treatment it is of importance to be aware of PDGF involvement in CNS tumor development (Fomchenko et al. 2007). Human adult SVZ NPCs and tumor cells from glioblastoma biopsies have been demonstrated by Vik-Mo *et al*. to share the capacity of forming neurospheres, of differentiation and of proliferation. Tumor cell markers used are also commonly applied NPC markers such as Sox2, CD133 and Nestin (Vik-Mo et al. 2011). This illustrates that there are similarities between NPCs and tumor cells and FT NPCs should be further characterized concerning protein expression of pluripotent genes to exclude risks of tumor formation and find an approach for identifying FT NPCs (Sundberg et al. 2011). Both adult human brain derived NPCs and NPCs from FT have been transplanted into rodents where the NPCs continued to proliferate and differentiate without forming tumors after 10-16 weeks (Olstorn et al. 2007, Varghese et al. 2009).

We also found that the NPCs in FT were distributed throughout the whole FT which demonstrates that NPCs are not restricted to a certain area in the FT and can be harvested independent of where the tissue is surgically obtained from within the FT. The NPCs were both present in the ependymal layer around the central canal and clustered more deeply. To our surprise we also detected many cells which were Sox2 positive *in vivo*. These findings make FT a possible source for NPC harvesting.

In conclusion, we and other groups have detected NPCs in the human FT (Varghese et al. 2009, Jha et al. 2012) which may act as a reservoir of NPCs for future NPC transplantations.

5.5 CONCLUDING REMARKS

The CNS has previously been regarded as an immune-privileged site, a concept that modern neuroscience has challenged during the last two decades. Neuroinflammation occurs in numerous diseases such as MS, neurodegenerative disorders, stroke as well as in neurotrauma and its role in the pathology of these conditions is well documented in the literature. However, the specific impact of inflammatory mediators on neural stem/progenitor cells is less well studied which is the rationale for the present study. It is probably crucial to understand the effects of inflammatory mediators on NPCs in order to develop successful cell therapies based on endogenous or transplanted cells to the CNS.

In this study we demonstrate that it is possible for a NPC to acquire a more "immune-like" phenotype which is followed by activation of TLR receptors and cytokine release. These findings suggest the novel concept that NPCs actually contribute to the inflammatory process. However the implication of these findings needs further studies.

The diversity of NPC response to inflammation presented in this thesis also includes changes of NPC fate due to their origin within the CNS. During healthy conditions the SVZ contains NPCs with neuronal features while NPCs from spinal cord are more prone to differentiate into a glial linage. During an inflammatory state the fate of the NPCs is skewed. The NPCs from spinal cord, where inflammation arises, change their fate towards a neuronal linage.

We found that inflammation-induced changes of NPC proliferation and differentiation were independent of the level of active inflammation indicating that any inflammatory event in the CNS may cause a wider damage that previously expected.

We also characterized NPCs in the FT in a large number of patients of various ages. We conclude that NPCs are numerous and widespread in the FT.

Future treatment strategies using NPCs may involve non-invasive manipulation of endogenous NPCs and/or transplantation of NPCs. To successfully do this a wider knowledge concerning aspects of NPCs' features must be achieved. Some of these features are presented in this thesis and hopefully our findings will contribute to the understanding of NPCs' plasticity and thereby shorten the distance between NPC based therapy and the patient.

6 CONCLUSIONS

I. NPCs in the SVZ express TLR receptors constitutively and when stimulated by experimental inflammation the NPCs may synthesize pro-inflammatory cytokines.

II. NPCs isolated from different part of the CNS vary in gene expression. Under basal conditions SVZ NPCs express a larger neurogenicity than spinal cord derived NPCs. After neuroinflammation the gene expression is changed. In the spinal cord NPCs genes regulating oligodendrogenesis are down regulated whereas neurogenesis related expression is increased. These changes are reflected in the functional outcome of cell differentiation.

III. Stemness and fate regulatory genes are affected in areas where the level of ongoing inflammation is low. Also in the less inflamed area NPCs fate is changed.

IV. The human FT harbors cells with NPC features. These cells can be propagated also from older donors. These immature cells from FT respond to growth factors and can be differentiated to glia and neurons. There is a rich abundance of NPCs in all parts of the FT.

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